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(54) Title: FUNCTIONAL HEAVY CHAIN ANTIBODIES, FRAGMENTS THEREOF, LIBRARY THEREOF AND METHODS OF PRODUCTION THEREOF

(57) Abstract: The present invention relates to functional heavy chain antibodies, functional single domain heavy chain antibodies, functional VH domains, or functional fragments thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45, and comprising an amino acid at position 103 independently chosen from the group consisting of R, G, K, S, Q, L, and P, and optionally an amino acid at position 108 independently chosen from the group consisting of Q, L and R, said positions determined according to the Kabat numbering.

FUNCTIONAL HEAVY CHAIN ANTIBODIES, FRAGMENTS THEREOF, LIBRARY THEREOF AND METHODS OF PRODUCTION THEREOF

Field of invention

The present invention relates to functional heavy chain antibodies, fragments thereof and a library thereof. It also relates to methods for producing functional heavy chain antibodies, fragments thereof and a library thereof. It further relates to uses of functional heavy chain antibodies, fragments thereof and a library thereof.

Background to the invention

The IgG isotype is the most abundant immunoglobulin found in sera. In all mammals, it is composed of two identical heavy (H) chains and two identical light (L) chains. Immunoglobulins harbouring this structure are therefore designated four-chain immunoglobulins. The H-chain of a 4-chain immunoglobulin contains 4 domains and a hinge region in between the second and third domain. The L-chain has two domains. The N-terminal domains of both the L- and H-chain are more variable in sequence than the remaining domains and are known as V-domains (VL and VH respectively). Three loops within the VH and three loops within the VL juxtapose in the paired VH-VL domains and constitute the antigen-binding site. The loops are hypervariable in sequence and named CDR for Complementarity Determining Region. A description of the general structure of a 4-chain Immunoglobulin is provided in "Immunology" Roitt I. *et al.*, Ed. MEDSI/ Mc GRAWHILL.

Much of the antigen binding diversity and the success of antibodies to generate a tight antigen binder against virtually all possible foreign substances, comes from the random pairing of one out of thousands of possible VHs with one out of thousands possible VLs. The second domain of the L-chain, having a more conserved sequence and denoted CL, is associated with the second domain of the H-chain (CH1) that has also a conserved sequence.

A pathological disorder in humans, known as heavy chain disease, is characterised by the presence of antibodies in the serum that do not contain L-chains. Moreover, these antibodies lack important parts of their VH and CH1 as well, although the missing VH and CH1 regions can vary widely among different HCAb (Heavy Chain Antibody). The deletions in the H-chain are due to deletions of the rearranged H-chain involving part of the VH and the CH1 domain. These antibodies no longer recognise antigen since the VL is absent and large parts of the VH is absent too. The HCAb can be secreted from the B-cells because the chaperone proteins (such as BIP) that associate with the CH1 retain the H chain in the endoplasmic reticulum until BIP is replaced by the L-chain. In absence of the CH1 polypeptide domain, the BIP can no longer retain the truncated H-chain in the endoplasmic reticulum, and the L-chain cannot bind either resulting in the fact that the H-chains are immediately secreted as homodimers.

Similar non- functional HCAbs were also reported to emerge in mouse monoclonal cell lines.

In sera of Camelidae (camels, dromedaries and llamas) we found the presence of the 4-chain immunoglobins and, in addition, of large amounts of functional HCAbs. These functional HCAbs have been described in European Patent Application N° 0656946 and in various publications including Hamers-Casterman *et al.* (1993), Vu *et al.* (1997) and Muyldermans *et al.* (2001). They are distinct from the human / mouse HCAbs present as a result of the pathological stage, in several respects. Firstly, they are functional in antigen binding. In this respect the HCAbs found in Camelidae are functional normal immunoglobulins. Secondly, in Camelidae, the entire CH1 domain is missing, and the V domain is intact but HCAbs have a sequence that deviates at a few sites from normal VH sequences. Said functional HCAb occur as a homodimeric molecules.

The CH1 is however encoded in the germline of all γ-genes in dromedaries (and llama) and is removed from the mRNA coding for the functional HCAbs by a splicing of the 3' end of the V-exon with the 5' end of the hinge exon. Thus, the CH1 is part of

the intron and is no longer recognized as an exon because of a single point mutation of the consensus splicing signal sequence. Llama and dromedary carry the same point mutation at the former CH1 exon and this finding indicates that these γ-genes emerged before the llama and camels diverged from each other. The different splicing activity of the mRNA is not an alternative splicing as all mRNA is spliced according to this scheme. Hence these γ-genes will always lead to a H-chain with its CH1 removed. Other γ-genes are used to produce the common H-chain with a CH1 domain.

The V-domain of the H-chain of functional HCAb (referred to as VHH, for Variable domain of the H-chain of a normal, i.e. immunologically-active HCAb) is expected to acquire adaptations versus the VH (i.e. V-domain of H-chain of conventional four-chain antibody) in the regions that are no longer contacting the VL (or the CH1) domain and in those participating in antigen binding (i.e. the paratope).

For instance, Clothia et al. (1985) have indicated in the above-referenced publication that crystallographic data revealed that conserved Val 37, Gly 44, Leu 45 and Trp 47 are clustered in space in a conventional 4-chain IgG and make important hydrophobic contacts with the VL. They added that the VH amino acids Gln 39, Tyr 91, Trp 103 and Glu 105 are also recognized as important for VL association. Desmyter et al (1996) further observed that the surface of the VHH domain which is present in camelidae and which corresponds to the VH side of conventional IgG which interacts with a VL is significantly reshaped in the camelid VHHs. In the present invention, the numbering of the amino acid residues is given by reference to the Kabat numbering (Kabat E, 1991) which is used in accordance with the Kabat database available at http://www.bioinf.org.uk/abs.

The most frequently occurring amino acid residues at twelve VH locations known to interact with VL have been determined for 332 vertebrate VH segments. It is mentioned that for the purpose of the present invention, the protein domain of the variable heavy polypeptide chain is referred as "VH" and the corresponding DNA is designated VH-D-J as it is assembled from a VH germline, a diversity D minigene

and a J minigene. In fact the CDR3 and FR4 are not encoded by the VH, but they are provided by D and J minigene that are recombined with the VH or (VHH) germline.

For comparison, the amino acid consensus has been deduced for 42 dromedary germline VHH sequences at the corresponding locations. The preferred amino acid residues at four positions (39, 43, 60 and 91, Kabat numbering) is invariable in VH and VHH. In contrast, at four other sites (33, 35, 50 and 58) neither VH nor VHH sequences reveal a pronounced amino acid preference. At the latter VH sites, the possible contact with the VL is dependent on the actual angle between VH and VL domains, and this explains the observed amino acid degeneracy. The only crucial differences between VH and VHH proteins in this area concern position 37, 44, 45 and 47. These are highly conserved amino acid residues among VH phenotypes (i.e. Val37, Gly44, Leu45 and Trp47), but in the VHH, the inventors observed most frequently Phe37 (or Tyr), Glu44, Arg45 (or Cys), and Gly47 (or Leu). These comparisons substantiate previous identifications of camel VHH-specific "hallmark" residues that arise in response to the absence of the L-chain.

From the results published by Nguyen et al. (2000), it is apparent that VHH and VH genes are imprinted in the dromedary genome. The VH and VHH genes are most likely residing in the same locus. It was noticed that the VH and VHH germline genes use the same D and J genes with the H-chain of conventional 4-chain antibodies. By PCR, around 50 VH and around 40 VHH germline genes were identified in dromedary. Each PCR fragment contains a leader signal exon and a V-exon, that ends where the CDR3 should start. The CDR3 and FR4 are provided by the recombined D-J segments. The VH germline segment harbours codons for Val37, Gly44, Leu45 and Trp47, and the VHH germline minigenes possess the Phe37 (11x) Tyr37 (30x) or in one single case Val37; Glu44 or Gln44 (8x); Arg45 (37x) or Cys45 (5x) and Gly47 (6x) or Leu47 (24x) or Trp47 (8x) or Phe47 (3x). In addition, these VHH germline- genes contain always (except 1) a Cys codon at position 45 or at the CDR1 region (codon 30,32 or 33). Based on the length of the CDR2 (16 or 17 amino

acids in size) and the location of the extra Cys, the VHH germline segments were grouped in subfamilies. Some subfamilies had several members while others are much scarcer in the genome. However, it should be noted that the frequency of occurrence of these VHH germline genes in expressed HCAb is not at all related to their frequency of occurrence in the genome. The Cys at position 45 or around the CDR1 is normally maintained in the rearranged VHH-D-J segments, and these rearrangements products have also acquired an extra Cys in the CDR3. Likewise, VHH-D-J rearrangements that were unable to generate an extra Cys in their CDR3 will apparently knock out the Cys45 or Cys in the CDR1 region probably by somatic hypermutation or by B-cell receptor editing. B cell receptor editing is a mechanism by which an upstream unrearranged V-segment is recombined into an existing V-D-J recombination product, that was most likely not functional, or recognizing a self antigen.

For dromedary, the VHH domains carry also longer CDR3 than that of the VH domains (average length 17-18 versus 9). Three possibilities can be envisaged to generate a longer CDR3. The VHH may uses two or more D minigenes, however, this is unlikely in view of the necessity to recombine two minigenes with a different recombination signal sequence (the 12-23 spacer rule). Alternatively, a more active terminal deoxynucleotidyl transferase during the D-J or V-D-J recombination might add several non-template encoded nucleotides. Finally, it can not be excluded that the length difference is only due to selection in which the fraction of VHH domains with long CDR3 or the VH domains with short CDR3 is much more likely to become functional to interact with antigen. A combination of the two latter explanations might also be relevant.

It has been proposed repeatedly that the presence of the VHH hallmarks at positions 37, 44, 45 or 47 or the substitution of the VH into the VHH hallmarks can lead to the formation of soluble single-domain antibody fragment. Of these, the amino acid at position 45 was considered crucial as the substitution of Leu45 of a human VH domain by Arg45 rendered the isolated domain more soluble. This camelised human

VH adopts a properly folded immunoglobulin structure (Riechman, 1996). Rearrangement of the former VL interface in the solution structure of a camelised, single domain VH antibody).

However, work of Chothia et al. (1985) revealed that amino acids of VH at position 35, 37, 39, 44, 45, 47, 91, 93 encoded by the VH gene segment, 95, 100, 101 as part of the CDR3, and 103, 105 encoded by the J gene segment are the key participants for the VL interaction. Of these, amino acids 37, 45, 47 differ largely between VH and VHH. Position 103 is occupied by a conserved Trp that is well buried in the VH-VL complex and provides the largest contact surface area with the VL after Leu45 and Trp47 (Figure 2 in Chothia et al.). As this Trp103 is encoded by the J gene and as the J gene is used in common in the VH-D-J and VHH-D-J recombination, it is logical to expect Trp at position 103 in VHH's as well. Since the VH-VL association is mediated by hydrophobic interactions, it is also clear that the substitution of the large aromatic and hydrophobic Trp 103 residue by the charged and hydrophilic Arg will prevent the association with a VL, and that of the surrogate light chain as well. WO92/01787 claims a single chain variable domain, being a synthetic variable immunoglobulin heavy chain domain, in which one or more of the amino acid residues at position 37, 39, 45, 47, 91, 93 or 103 is altered, whereby the tryptophan at position 103 is changed into glutamate, tyrosine or threonine. However, there is no indication that a substitution of tryptophan at 103 alone by arginine, glycine lysine, proline or serine would be sufficient to obtain a functional heavy chain antibody, neither that this mutation could compensate for the absence of a charged amino acid or a cysteine at position 45, nor that said mutation may result in an increased solubility of a single domain heavy chain antibody fragment.

It is known in the art that the production of antibodies, for example by bacterial overexpression techniques, by phage display libraries, is technically difficult due to the antibody or fragments thereof being poorly expressed, insoluble, mis-folded. It is also known that the screening of antibody libraries is restricted to those which are

soluble, so excluding a large fraction of antibodies with potentially active antigen binding regions. Thus binders which might be therapeutically useful would be precluded from screening. There is a need by researchers involved in discovering new therapeutic agents for a method for producing functional antibodies and fragments thereof. There is a need by researchers involved in discovering new therapeutic agents for antibody libraries comprising functional antibodies. There is a need by researchers involved in discovering new therapeutic agents for methods to functionalise antibodies.

Summary of the invention

One embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising, an amino acid which is neither a charged amino acid nor a C at position 45, and comprising an amino acid at position 103 independently chosen from the group consisting of R, G, K, S, Q, L, and P, and optionally an amino acid at position 108 independently chosen from the group consisting of Q, L and R, said positions determined according to the Kabat numbering.

Detailed description of the invention

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 13 (RGQGTQ) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 14 (RGKGTQ) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising SEQ ID NO: 15 (VXXXXXXGLXW) according to Figure 6, wherein X is any amino acid, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 16 (LGQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 17 (QGQGTGVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 18 (PGQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 19 (SSQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1 to 10 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 3, 5, 7 or 9 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 2, 4, 6, 8 or 10 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 2, 3, 4 or 5 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 6, 7, 8, 9 or 10 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 5, 6, 7, 8 or 9 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 3, 7, 9 or 10 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 2, 5, 8, 9 or 10 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 3, 4, 5, 6 or 7 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 4, 6, 7, 8 or 9 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 20 to 79 according to Figure 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 1.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 2.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 3.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 4.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 5.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 6.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 7.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 8.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 9.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 10.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 20.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 21.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 22.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 23.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 24.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 25.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 26.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 27.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 28.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 29.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 30.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 31.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 32.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 33.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 34.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 35.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 36.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 37.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 38.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 39.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 40.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 41.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 42.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 43.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 44.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 45.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 46.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 47.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 48.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 49.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 50.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 51.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 52.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 53.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 54.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 55.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 56.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 57.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 58.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 59.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 60.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 61.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 62.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 63.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 64.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 65.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 66.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 67.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 68.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 69.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 70.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 71.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 72.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 73.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 74.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 75.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 76.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 77.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 78.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising SEQ ID NO: 79.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from camel.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from human.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from mouse.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from rabbit.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from goat.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from kangaroo.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein said macromolecule is derived from sheep.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of the macromolecules above wherein

said macromolecule is derived from any vertebrate species other than camel, human and mouse.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of the macromolecules above, as an artificial mutant.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of according to any of the macromolecules above, as a peptide homologue of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof.

By "homologue" as meant herein is an amino acid sequence which is at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5% identical to the amino acid sequences of the present invention. By a polypeptide with an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence of the present invention, it is intended at the amino acid sequence of the polypeptide is identical to the reference sequence, except that it may have up to 5% of its amino acids deleted or substituted compared with the reference sequence, or, except that the sequence may have amino acid insertions up to 5% of the total number of amino acids in the reference sequence. As a practical matter, whether any particular peptide is at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5% identical to the amino acid sequences of the present invention can be determined using known algorithms.

Another embodiment of the invention is a polypeptide comprising a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, as described above.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, as described above wherein one or more amino acids are derivatized.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above wherein said macromolecules are dimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above wherein said macromolecules are trimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above wherein said macromolecules are multimeric.

Another embodiment of the invention is a method to functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof by replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and optionally replacing the amino

acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

Another embodiment of the invention is a method to functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof by replacing the amino acid at position 103 with R, said position determined according to the Kabat numbering.

Another embodiment of the invention is a method to humanize and functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof, said method comprising replacing the amino acid at position 45 with L, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position 44 with G and/or the amino acid at position 47 with W, and replacing of amino acid at position 103 with R, said position determined according to the Kabat numbering

Another embodiment of the invention is a method to humanize and functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof, said method comprising replacing the amino acid at position 45 with L, replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position 44 with G and/or the amino acid at position 47 with W, and optionally replacing the amino acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from human or mouse.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from human.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from mouse.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from rabbit.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from goat.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from sheep.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from rat.

Another embodiment of the invention is a method according to the above methods, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from any vertebrate species other than human and mouse.

Another embodiment of the invention is a method to humanize a functional camelid heavy chain antibody, a functional camelid single domain heavy chain, a functional camelid VHH domain or a functional fragment thereof, said method comprising replacing the amino acid at position 45 with L, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position 44 with G and/or the amino acid at position 47 with W, said positions determined by the Kabat numbering.

Another embodiment of the invention is a method to camelize a functional heavy chain antibody, a functional single domain heavy chain, a functional VH domain or a functional fragment thereof, said method comprising replacing the amino acid at position 45 with an amino acid independently chosen from the group consisting of L, V and P, replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and replacing the amino acid at position 37 with F, the amino acid at position 44 with G, the amino acid at position 47 with W, and amino acid at position 103 with R, and optionally replacing the amino acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtainable by the methods above.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtained by the methods above.

Another embodiment of the invention is a functional humanized camelid heavy chain antibody, a functional humanized camelid single domain heavy chain, a functional

humanized VH domain or a functional fragment thereof, obtainable by the methods above.

Another embodiment of the invention is a functional humanized camelid heavy chain antibody, a functional humanized camelid single domain heavy chain, a functional humanized VH domain or a functional humanized fragment thereof, obtained by the method above.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to above 4 paragraphs, as an artificial mutant.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to above 5 paragraphs, as a peptide homologue of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof.

Another embodiment of the invention is a polypeptide comprising a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to according to above 6 paragraphs.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to the above 7 paragraphs which recite said macromolecules, or a polypeptide according the above paragraph wherein one or more amino acids is derivatized.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtained by the methods as defined above wherein said macromolecules are dimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtainable by the methods as defined above wherein said macromolecules are dimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtained by the methods as defined above wherein said macromolecules are trimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtainable by the methods as defined above wherein said macromolecules are trimers.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtained by the methods as defined above wherein said macromolecules are multimeric.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof obtainable by the methods as defined above wherein said macromolecules are multimeric.

Another embodiment of the invention is a library, comprising one or more functional heavy chain antibodies, functional single domain antibodies, functional VH domains, or functional fragments thereof as defined above.

Another embodiment of the invention is a method to make a library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or a functional fragment thereof, comprising the steps:

- introducing a restriction enzyme recognition site in the coding region of the framework 4 region of a VH chain, whereby the cutting site of said restriction enzyme recognition site is situated in the CDR3 region,
- cutting the nucleic acid molecule comprising said coding sequence with said restriction enzyme,

- ligating a double stranded primer to the VH encoding nucleic acid molecule, restoring the CDR3 and so introducing an R amino acid at position 103, said position determined by the Kabat numbering, and
- amplifying the ligated fragments.

Another embodiment of the invention is a method according to the above, whereby said restriction enzyme cut is situated within the last two codons of the CDR3 coding region.

Another embodiment of the invention is a method according to the above, whereby said restriction enzyme creates a GA 3' sticky end by cutting before the first nucleotide of the codon coding for amino acid position 101 and after the second nucleotide of codon coding for amino acid position 101 on the complementary strand, said positions determined according to the Kabat numbering.

Another embodiment of the invention is a method according to the above, whereby said restriction enzyme cut is situated within the last codon of the CDR3 coding region.

Another embodiment of the invention is a method according to the above, whereby said restriction enzyme is creating a CA-3' sticky end by cutting before the second nucleotide of codon coding for amino acid position 102 and after the third nucleotide of codon 102 on the complementary strand, said position determined according to the Kabat numbering.

Another embodiment of the invention is a method according to the above, whereby said restriction enzyme is chosen from the group consisting of BpmI, Eco57I, BsgI, Smu I, Fau I, Bse RI, and Bfi I.

Another embodiment of the invention is a method to make a library comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, comprising a step of amplification of nucleic acid strands encoding a repertoire of immune or non-immune VHH antibodies, using a framework 1 specific primer as forward primer, and a back primer which anneals to said nucleic acid strands such that its 3'-terminal three nucleotides are positioned over the codon of the nucleic acid strands which encode amino acid position 103, the reverse-complement of said 3'-terminal three nucleotides encoding R103, K103, Q103, F103, P103, G103 or S103, said position determined according to the Kabat numbering.

Another embodiment of the invention is a method to make a library comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, comprising a step of

amplification of nucleic acid encoding a repertoire of immune or non-immune VHH antibodies or fragments thereof, using a framework 1 specific primer, as forward primer, and using one or more of SEQ ID NOs: 80 to 88 according to Figure 6 as back primers.

Another embodiment of the invention is a library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, obtainable by the method according to any of claims as defined above.

Another embodiment of the invention is a library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, obtained by the method as defined above.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from human or mouse.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from camel.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from sheep.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from human.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from mouse.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from rat.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from goat.

Another embodiment of the invention is a library as defined above wherein the methods use a single domain heavy chain library from any vertebrate species other than camel, human or mouse.

Another embodiment of the invention is a heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtained by the method as defined above for use in immunoassays.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 21bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 42bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a

functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 63bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct

comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 83bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 150bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 240bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences,

wherein the coding sequence comprises a DNA sequence of at least 300bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a nucleic acid comprising a DNA sequence encoding a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a nucleic acid having a nucleotide sequence which is at least 65% identical to the sequence as defined above.

Another embodiment of the invention is a vector comprising a nucleic acid sequence as defined above.

Another embodiment of the invention is a host cell comprising an integrated or episomal copy of a nucleic acid molecule as defined above, or a vector as defined above.

Another embodiment of the invention is the host cell of as used above, wherein said host cell is a yeast, bacterial, insect, fungal, plant or mammalian cell.

Another embodiment of the invention is a method for producing a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, comprising:

(a) culturing host cells comprising a nucleic acid as defined above, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, or nucleic acid as defined above for the preparation of a medicament.

Another embodiment of the invention is a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, or nucleic acid as defined above for the preparation of a medicament for the treatment of a disease related to asthma, rhinoconjunctivitis, allergic disorders, acute allograft rejection, Crohn's disease and ulcerative colitis.

Another embodiment of the invention is a pharmaceutical composition comprising a functional heavy chain antibody, a functional single domain heavy chain antibody, a

functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, or nucleic acid as defined above, optionally in combination with a suitable excipient.

Another embodiment of the invention is the use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, or nucleic acid as defined above in the diagnosis of a disease related to asthma, rhinoconjunctivitis, allergic disorders, acute allograft rejection, Crohn's disease and ulcerative colitis.

Another embodiment of the invention is the use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above for the purification of a protein.

Another embodiment of the invention is the use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above for the purification of a protein.

Another embodiment of the invention is a kit for the diagnosis of a pathological condition or a susceptibility to a pathological condition in a subject comprising a nucleic acid as defined above, a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above.

Another embodiment of the invention is a method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of :

(a) determining the presence or absence of a mutation in the nucleic acid as defined above, including mutations in the genomic and regulatory sequences of said nucleic acid, in a biological sample, and

(b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or absence of said mutation.

Another embodiment of the invention is a method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of :

(a) determining the presence or amount of the nucleic acid as defined above or expression of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment as defined above or a polypeptide as defined above in a biological sample, and,

(b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of said nucleic acid or expression of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, functional fragment thereof or polypeptide.

Another embodiment of the invention is a drug screening assay for screening test compounds which interact with a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above, comprising:

(a) combining a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof as defined above or a polypeptide as defined above with a test compound, under conditions which allow for interaction of the test compound to said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, functional fragment thereof or polypeptide, to form a complex, and,

(b) detecting the formation of a complex, in which the ability of the test compound to interact with the said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, is indicated by the presence of the test compound in the complex.

Another embodiment of the invention is the product or compound identifiable by the assay as defined above.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 80 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 81 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 82 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 83 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 84 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 85 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 86 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 87 according to Figure 6.

Another embodiment of the invention is nucleic acid comprising the sequence SEQ ID NO: 88 according to Figure 6.

Another embodiment of the invention is a nucleic acid having a nucleotide sequence which is at least 65% identical to the sequence as defined above.

Another embodiment of the invention is the use of a nucleic acid as defined above in a method to produce one or more functional heavy chain antibodies, functional single domain heavy chain antibodies, functional VH domains, or functional fragments thereof.

The antibodies of the above embodiments are functional and as such exhibit improved properties, for example, expression levels, stability, affinity and solubility over antibodies in which the characterising features absent. It is known in the art that the production of antibodies, for example by bacterial overexpression techniques, in phage display libraries, for screening libraries, is difficult due to the properties of the antibody or fragments thereof.

Surprisingly, the inventors have found that a heavy chain, carrying a mutation at position 103, possibly combined with a mutation at position 108 behaves as a functional heavy chain antibody (HCAb), even if it does not carry the VHH hallmark amino acids at positions 37, 44, 45 and 47. In particular, the inventors have found that a mutation wherein the amino acid residue corresponding to position 103 (Kabat numbering) is mutated to an amino acid selected among arginine, glycine, proline, serine, leucine, glutamine or lysine can compensate for the loss of the critical hallmark amino acid at position 45, whereby the charged amino acid or the cysteine at position 45, may be changed in any other amino acid, but preferably into a leucine. Prior to an aspect of the present invention, heavy chains having said amino acids at positions at 45 and 103 were considered by the person skilled in the art as part of a classical 4-chain antibody complex (Harmsen et al. 2000) and not as a functional

heavy chain antibody. Moreover, for some antibodies having residues which characterise one aspect of the present invention, the presence of a light chain has been described (Anker et al., 1990; Chukwuocha at al., 1999), clearly demonstrating that these antibodies were not functional heavy chain antibodies.

Surprisingly, the inventors were able to show that an amino acid at position 103 selected among arginine, glycine, proline, serine or lysine increases the solubility of the heavy chain, while it may disrupt the possibility to interact with a light chain. Therefore, such a heavy chain molecule behaves like a functional heavy chain antibody, even without the hallmark amino acid for the functional HCAb at position 45.

The invention relates to a functional HCAb, comprising an amino acid, which is neither a charged amino acid nor a cysteine at position 45, and comprising an amino acid chosen from the group consisting of arginine (R), glycine (G), lysine (K), serine (S) and proline (P) at position 103, possibly combined with glutamine (Q) at position 108 according to the Kabat numbering. In one embodiment of the invention, the amino acid at position 45 is a L. In another embodiment, the amino acid at position 103 is an R. In another embodiment, the amino acid at position 103 is an R and the amino acid at position 108 is a Q.

In one embodiment of the invention embodiment, the functional HCAb or fragment thereof according to the invention is an artificial mutant. An artificial mutant, as used here, means that the change is introduced intentionally and differs from the sequence found in the natural situation. Said artificial mutant may be derived from a variable domain (designated VH) of a heavy polypeptide chain of an immunoglobulin wherein the amino acid residue corresponding to position 103 (Kabat numbering) is mutated to an amino acid selected from the group consisting of R, G, K, S and P, possibly in combination with a mutation wherein the amino acid residue corresponding to position 108 (Kabat numbering) is mutated to Q. In another embodiment, said artificial mutant is derived from a variable domain (designated VH) of a heavy polypeptide chain of an immunoglobulin wherein the amino acid residue corresponding to position 103 (Kabat numbering) is mutated to arginine. In another

embodiment, said artificial mutant is derived from a variable domain (designated VH) of a heavy polypeptide chain of an immunoglobulin wherein the amino acid residue corresponding to position 103 (Kabat numbering) is mutated to arginine and the amino acid residue corresponding to position 108 (Kabat numbering) is mutated to glutamine.

Alternatively, said artificial mutant may be derived from a variable domain (designated VHH) of a heavy polypeptide chain of a heavy chain antibody wherein the hallmark amino acid residue at position 45 is mutated to a Leucine, possibly in combination with a mutation of one or more of the other hallmark amino acids at position 37, 44 and 47 to Val37, Gly44 and Trp47. One embodiment is an artificial mutant wherein all hallmark amino acids at position 37, 44, 45 and 47 are mutated to Val37, Gly44, Leu45 and Trp47.

An artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence RGQGTQ (SEQ ID N°13) or alternatively sequence RGKGTQ (SEQ ID N°14).

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VHH domain and encompasses within this polypeptide sequence VXXXXXXGLXW (SEQ ID N°15), whereby X can be any amino acid.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence RGQGTQ (SEQ ID N°13), wherein R of said sequence is at position 103 according to the Kabat numbering. Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence RGKGTQ (SEQ ID N°14), wherein R of said sequence is at position 103 according to the Kabat numbering.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VHH domain and encompasses within this polypeptide sequence VXXXXXXGLXW (SEQ ID N°15), wherein V of said sequence is at position 37 according to the Kabat numbering, whereby X can be any amino acid.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence LGQGTQVTVSS (SEQ ID N°16), wherein L of said sequence is at position 103 according to the Kabat numbering.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence QGQGTGVTVSS (SEQ ID N°17), wherein L of said sequence is at position 103 according to the Kabat numbering.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence PGQGTQVTVSS (SEQ ID N°18), wherein L of said sequence is at position 103 according to the Kabat numbering.

Another artificial mutant according to the invention comprises a polypeptide sequence derived from the VH domain and encompasses within this polypeptide sequence SSQGTQVTVSS (SEQ ID N°19), wherein L of said sequence is at position 103 according to the Kabat numbering.

Said SEQ ID NOs: 13 to 19 are those cited in Figure 6.

The artificial mutant according to the invention is derived from a VH domain, or a VHH domain, meaning that in accordance with the present invention it can be isolated from said domain by introducing said mutations, or it can be synthesized, including chemically synthesized or expressed especially by recombinant techniques, including in host cells, starting from the knowledge of the polypeptide sequence of said VH domains or VHH domains and the position of the mutations to be introduced. More generally, it can be prepared by any method made available for the preparation of polypeptide chains.

The polypeptide chain derived from the VH gene and having the features as defined here above may be obtained by methods involving site-directed mutagenesis or PCR (using primers carrying said the mutation), starting from a conventional VH-D-J gene, especially one obtained from a library. A relevant method is that described by

Hemsley et al (1989). The present invention thus provides the possibility to generate soluble mutant single domain antibody fragments that originate from a VH-D-J gene. The method of Hemsley, however, requires that the sequence of the gene to be mutated is known, at least in the region where the mutation has to be introduced. As a position to be mutated, residue 103, is adjacent to a variable region, the method of Hemsley et al. (1989) is not suitable for the introduction of a mutation in VHs with unknown variable regions, and an adapted method has to be applied in this case.
Alternatively, the polypeptide chain derived from the VHH and having the features as defined here above may be obtained by methods involving site-directed mutagenesis or PCR (using primers carrying said the mutation), starting from a HCAb VHH, especially one obtained from a library. As these mutations are situated in a conserved framework region, the method of Hemsley et al. (1989) can be applied. In that case, the present invention provides the possibility to humanize single domain antibody fragments that originate from a VHH-D-J gene. Humanization may comprise the replacement of one or more of the VHH hallmark amino acids at position 37, 44, 45 and 47 into the conserved human residues Val37, Gly44, Leu 45 and Trp47. However, to introduce the compensating mutation at position 103, the sequence of the adjacent variable region should be known, as discussed above.
The invention relates further to a method to solubilize single domain heavy chain fragment derived from conventional 4-chain immunoglobulins. Indeed, the inventors have shown that the presence at position 103 of a hydrophilic amino acid residue, especially of a residue selected among arginine, glycine, proline, serine or lysine renders the resulting polypeptide derived from the VH more soluble with respect to the same polypeptide having a tryptophan residue at position 103. This effect may even be enhanced by replacing the amino acid at position 108 by a glutamine.

Another aspect of the invention is a method to "humanize" a camelid heavy chain antibody, said method comprising at least the replacement of the camelid hallmark amino acid at position 45, possibly combined with a replacement of one or more of the other hallmark amino acids at position 37, 44 and 47. Humanizing, as used here,

means that one or more of the camelid hallmark amino acids in the HCAb are replaced by their human counterpart as found in the human consensus sequence, without that said heavy chain antibody is losing its typical character, i.e. the humanization does not significantly affect the antigen binding capacity of the resulting HCAb or fragment thereof.

Another aspect of the invention is a functional single domain heavy chain antibody fragment, obtainable by the method according to the invention. Still another aspect of the invention is a functional single domain heavy chain antibody fragment, obtained by the method according to the invention. Still another aspect of the invention is a functional humanized single domain heavy chain antibody fragment, obtainable by the method according to the invention. Still another aspect of the invention is a functional humanized single domain heavy chain antibody fragment, obtained by the method according to the invention.

Another aspect of the invention is a library, comprising one or more functional single domain heavy chain antibody fragments according to the invention. In one embodiment of the present invention, said library comprises at least 100, or at least 1000, or at least 10 000, or at least 100 000, or at least 1 000 000 functional single domain heavy chain antibody fragments. Such a library has the advantage that it is composed of soluble molecules, contrary to a possible library of classical VH molecules, which would be insoluble and non-functional (Nutall et al., 2000). Whereas for one single VH, the problem may be solved by denaturation, followed by refolding and resolubilization, this procedure is not possible in case of the creation of a complex library, especially not when said creation is followed by the selection of domains with varying antigen binding specificities. Indeed, generation of insoluble and therefore "sticky" scaffold protein during selection procedures can lead to false positives through non-specific binding to antigen by hydrophobic patches on the displayed domain. Several attempts have been made to overcome this aggregation problem (Pessi et al, 1993; Quiocho, 1993; Dimasi et al., 1997), but till now, the attempts of producing single domain VH libraries with acceptable solubility characteristics have not been successful. Therefore, the soluble single domain

heavy chain antibody fragment according to the invention allow for the first time the efficient construction of a library comprising functional single chain antibody fragments.

As the functional single domain heavy chain antibodies according to the invention do normally not occur in nature, such a library cannot be made by direct amplification of the messenger RNA, but has to be made by the use of mutagenic primers. Although in principle, a VHH backbone may be used as starting material, this has several drawbacks, as not only the camelid hallmark amino acids have to be mutated, but the compensating mutation at position 103 has to be introduced. For this reason, it is preferable to construct the library starting from a human VH mRNA library. From such a library, potent antigen binders may be retrieved. Contrary to the VHH situation, starting from a VH backbone, only the mutation at position 103 has to be introduced.

The problem to make such library is that the 3'-end primer used to amplify the VH by RT-PCR needs to be mutagenic for the codon 103, and the primer should extend for at least 4-5 nucleotides towards its 3'-end to anneal perfectly to the template. However, since this region is part of the CDR3, such a primer will contain too much degeneracies so that no DNA amplification will be achieved. As a consequence, making a functional single domain heavy chain library, according to the invention is far from obvious.

Therefore another aspect of the invention is a method to make a library, comprising at least one functional single chain library, according to the invention, comprising

- introducing a restriction enzyme recognition site in the coding region of the framework 4 region of a VH or VHH chain, whereby the cutting site of said restriction enzyme recognition site is situated in the CDR3 region
- cutting the nucleic acid molecule comprising said coding sequence with said restriction enzyme
- ligating a double stranded primer to the remaining V encoding nucleic acid molecules, restoring the CDR3 and framework 4 codons and introducing the 103 mutation in the framework 4

- amplifying the ligated fragments.

One embodiment comprises said method whereby the method is carried out on a pool of coding sequences, such as a pool of mRNA as well as on one isolated coding sequence. Another embodiment comprises said method whereby the method is carried out on one isolated coding sequence, and a synthetic library is generated by randomizing one or more codons of one or more of the CDR loops. Alternatively, a library may be generated by grafting camelid CDR loops on the mutated framework, comprising the 103R mutation.

In another embodiment, the restriction enzyme cut is situated within the last codon or within the last two codons of the CDR3 coding region. One embodiment comprises said method, whereby said enzyme is creating a blunt end at the CDR3 – framework 4 junction. Another embodiment comprises said method, whereby said enzyme creates a CA 3' sticky end by cutting before the second nucleotide of codon 102 and after the second nucleotide of codon 102, according to the Kabat numbering. Indeed, in most VH and VHH's, there is a conserved tyrosine (Y) at position 102. This amino acid is most frequently encoded by TAC. Another embodiment comprises said method, whereby said enzyme creates a GA 3' sticky end by cutting before the first nucleotide of codon 101 and after the second nucleotide of codon 101, according to the Kabat numbering. Indeed, in most human VH, there is a conserved aspartic acid (D) at position 101, a charged amino acid that is important for the CDR3 loop structure. This amino acid is encoded by either GAC or GAT. By creating

a GA 3' sticky end, the conserved codon may be restored by the ligation to the double stranded primer. In that case, the codon 102 may be either randomized or fixed, by ligation of the primer. One embodiment comprises said method above, whereby said restriction site is Bpm I. Another embodiment comprises said method, whereby said restriction site is Eco57 I. Another embodiment comprises said method, whereby said restriction site is Bsg I. An embodiment comprises said method, whereby said restriction site is Fau I. Another embodiment comprises said method,

whereby said restriction site is Smu I. Another embodiment comprises said method, whereby said restriction site is Bse RI.

Another embodiment comprises said method, whereby the restriction site is BfiI, introduced in such a way that the enzyme cuts at the CDR3 junction in the upper strand, and between the first and the second nucleotide of CDR3, adjacent to the framework 4 in the lower strand. In the latter case, the CDR3 and framework 4 regions may be restored by ligation with a double stranded primer consisting of the framework 4 coding region for the upper strand, and the complementary strand thereof, with either a TG 3' overhang, or a TG-3' overhang and an extra codon such as GTG, or TAC before the Trp103 codon.

Still another aspect of the inventions is a library obtainable by the invention, comprising one or more functional single domain heavy chain antibody fragments. Still another aspect of the invention is a library, obtained by the invention, comprising one or more functional single domain heavy chain antibody fragments.

In one embodiment, said library comprises at least 100, in another embodiment at least 1000, in another embodiment at least 10 000, in another embodiment at least 100 000, in another embodiment at least 1 000 000 functional single domain heavy chain antibody fragments.

Surprisingly, we have found that a significant fraction of the camelid antibodies comprises functional heavy chain antibodies according to the invention, contrary to what is assumed by the person skilled in the art. This significant fraction represent a new class of functional heavy chain antibodies. It would not be obvious, therefore, to a skilled artisan that a functional heavy chain antibody and/or a functional soluble single domain heavy chain antibody fragment can be isolated directly from a mRNA preparation from camelids, and this material can be used as starting material for the preparation of a functional soluble single domain heavy chain antibody fragment library according to the invention. As a consequence, another aspect of the invention is a library obtained by specific amplification and cloning of the new class of functional heavy chain antibodies described in this invention, and which have more

homology to human antibodies than the class of VHH with the hydrophilic residues in FR2. In order to obtain this new class of VHH from a repertoire of immune or non-immune antibodies, specific primers for amplification were designed, that anneal preferentially to genes encoding VHH with Arginine, Lysine, Glutamine, Phenylalanine, Proline, Glycine, Tryptophan or Serine as residue 103. To accomplish specific annealing the 3' site of the primer ends exactly at the first nucleotide of the codon coding for residue 103, which in the new class of VHH is different from the Tryptophan 103 containing VHH fragments.

The following primers were designed:

primer 1 (R103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/T/C/G)CG-3'

primer 2 (R103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (C/T)CT-3'

primer 3 (K103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC GCT GGA GAC GGT GAC CTG GGT CCC CTG GCC (T/C)TT-3'

primer 4 (Q103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GC(C/G) (C/T)TG-3'

primer 5 (L103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/G/C/T)AG-3'

primer 6 (F103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/G)AA-3'

primer 7 (G103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CCC CGG (A/G/C/T)CC-3'

primer 8 (S103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG (A/G/C/T)GA (A/G/C/T)GA-3'

primer 9 (P103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG CTG (A/G/C/T)GG-3'

primer 10 (Y103): 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/G)TA-3'

Specific amplification was carried out using a Framework 1 specific primer, that is 5' linked to a SfiI site, as forward primer, and a pool of primers 1-10 as back primers for the amplification of functional soluble single domain heavy chain antibody fragments according to the invention. The resulting material was cut with SfiI and NotI or BstEII and the resulting fragment is cloned into pHen4.

Still another aspect of the invention is the use of a functional heavy chain antibody according to the invention or a functional soluble single domain heavy chain antibody fragment according to the invention for the preparation of a medicament. Still another aspect of the invention is a pharmaceutical composition, comprising a functional heavy chain antibody, according to the invention, or comprising a functional soluble single domain heavy chain antibody fragment, according to the invention, optionally in combination with a suitable excipient. Indeed, antibodies may be used in the treatment of several diseases, such as, as a non-limiting example, asthma and rhinoconjunctivitis (Botger et al, 2002), allergic disorders (Babu et al, 2001), acute allograft rejection (Sollinger et al, 2001), Crohn's disease (Hommes et al, 2002) and ulcerative colitis (Gordon et al, 2002). The functional soluble single

domain heavy chain antibody fragment may have a significant advantage due to their small size and their solubility.

Another aspect of the invention is the use of a functional heavy chain antibody according to the invention or a functional soluble single domain heavy chain antibody fragment according to the invention in diagnosis. Diagnostic methods, using antibodies are known to the person skilled in the art and included, as a non-limiting example ELISA and RIA methods. The antibodies according to the invention do have several additional advantages in these assays, due to their stability and the fact that they can be fixed on a solid support without significant loss of activity. The latter characteristic makes them specially suitable for coating of surfaces, as may be desirable in several immunological detection techniques, including their use in microarrays.

Still another aspect of the invention is the use of a functional heavy chain antibody according to the invention or a functional soluble single domain heavy chain antibody fragment according to the invention in the purification of proteins and other molecules. Purification methods such as, as a non-limiting example, immunochromatography are known to the person skilled in the art. The antibodies according to the invention do have several additional advantages in such purification methods, due to their stability, that may guarantee a long lifetime of the purification carrier, and due to the fact that they can be fixed on a solid support without significant loss of activity.

DEFINITIONS

The following definitions are set forth to illustrate and define the meaning and scope of various terms used to describe the invention herein.

"Derivatized" as used herein in reference to a polypeptidic macromolecule means comprising derivatized amino acids. For example, homo-phenylalanine, citrulline,

and noreleucine are considered derivatized amino acids for the purposes of the invention. Derivatized amino acids also include imino acid residues such as proline and hydroxyproline. In addition, any amino acid representing a component of the variant proteins of the present invention, replaced by the same amino acid but of the opposite chirality, is considered derivatized. Thus, any amino acid naturally occurring in the L- configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D-amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability, and therefore are advantageous in the formulation of compounds which may have longer in vivo half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

In the preferred embodiment, the derivatized amino acids are in the (S) or L-configuration or the (S) or D-configuration. Derivatized amino acids may be used, for example, to prevent or retard in vivo degradations. Proteins including non-naturally occurring amino acids may be synthesized or in some cases, made recombinantly; see van Hest et al., FEBS Lett 428:(1-2) 68-70 May 221998 and Tang et al., Abstr. Pap Am. Chem. S218:U138-U138 Part 2 August 22,1999, both of which are expressly incorporated by reference herein.

Aromatic amino acids may be replaced with D- or L-naphylalanine, DM or L-Phenylglycine, D- or L-2- thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)- alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)- phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole(alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be regarded as derivatized when they are substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., -SO3H) threonine, serine, or tyrosine.

Other substitutions may include unnatural hyroxylated amino acids. Other derivatives may made by combining "alkyl" with any natural amino acid. The term "alkyl" as used herein refers to a branched or unbranched saturated hydrocarbon group of 1 to 24 carbon atoms, such as methyl, ethyl, n-propyl, isoptopyl, n- butyl, isobutyl, t-butyl, octyl, decyl, tetradecyl, hexadecyl, eicosyl, tetracisyl and the like. Alkyl includes heteroalkyl, with atoms of nitrogen, oxygen and sulfur. Preferred alkyl groups herein contain 1 to 12 carbon atoms. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amide linkage in any of the variant polypeptides can be replaced by a ketomethylene moiety. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased in vivo half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications of amino acids of variant polypeptides of to the present invention may include the following: Cysteinyl residues may be reacted with alpha-haloacetates (and corresponding amine), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues may also be derivatized by reaction with compounds such as

bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, P- chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7 -nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5 to 7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used, e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysinyl residues.

Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters e.g., as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate. Arginyl residues

may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2, 3-butanedione, 1 ,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group. The specific modification of tyrosyl residues per se is well-known, such as for introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane.

N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl- (4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-

dimethylpentyl) carbodiimide. Furthermore aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. Glutaminyl and asparaginyl residues may be frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

"Functional" in reference to a heavy chain antibody, a single domain heavy chain antibody, a VH domain or fragments thereof means that the same retains a significant binding (dissociation constant in the micromolar range) to its epitope, compared with its binding *in vivo*, and that it shows no or limited aggregation (soluble and non-aggregated above 1mg/ml), so allowing the use of the antibody as a binder.

"Functionalized" in reference to a heavy chain antibody, a single domain heavy chain antibody, or fragments thereof means to render said heavy chain antibody, a single domain heavy chain antibody, or fragments thereof functional.

By "*fragments thereof*" as used herein, is meant a portion corresponding to more than 95% of the sequence, more than 90% of the sequence of, more than 85% of the sequence of, more than 80% of the sequence of, more than 75% of the sequence of, more than 70% of the sequence of, more than 65% of the sequence of, more than 60% of the sequence of, more than 55% of the sequence of, or more than 50% of the sequence of.

"Coding sequence" is a nucleotide sequence, which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the complete coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. However, coding sequence as used here is not limited to

the complete coding sequence, but includes fragments thereof; such fragments are also indicated as *coding region*. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

"*Human hallmark amino acids*" as used herein in reference to the humanization of non-human antibodies are Val37, Gly44, Leu45, Trp47, positions determined according to the Kabat numbering.

"Nucleotide sequence", "DNA sequence", "nucleic acid molecule(s)" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog.

"Upper strand" of a DNA sequence is the strand that comprise the DNA version of the codons as they occur in the mRNA, *lower strand* is the strand with the anticodons, that is used as template to synthesize the mRNA.

"*VH domain*" as used herein means the variable domain of H-chain of a conventional four-chain antibody.

"*VHH domain*" as used herein means variable domain of the H-chain of a conventional, (i.e. immunologically functional) HCAb.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1-1 and 1-2: Nucleotide and translated amino acid sequences of wild type anti- b-lactamase VHH TEM04 (A) and anti- carcinoembryonic antigen VHH CEA71 (B).

Figure 2: Western blot analysis of R103W mutant and wild type VHH from (A) anti-β-lactamase VHH TEM04 and (B) anti- carcinoembryonic antigen VHH CEA71. From each clone three cultures were induced and used for analysis. As positive control the anti-lysozyme VHH cAblys3 was used.

Figure 3: Coomassie stained 15% SDS PAGE of IMAC purified R103W mutant and wild type VHH. The slower migrating product in the two lanes on the left is β-lactamase, which was complexed to the VHH.

Figure 4: Antigen binding determined by ELISA of VHH CEA71 wild type (WT) and R103W mutant.

Figure 5: Examination of the solubility characteristics of VHH CEA72 (wild type and R103W mutant) by determining the concentration of ammonium sulfate at which the fragment started to precipitate. The amount of VHH in the supernatant was measured (A) by protein content with OD280, or (B) by antigen binding in ELISA.

Figure 6-1, 6-2, 6-3: SEQ ID NOS: 1 to 10 and 20 to 79: sequences of heavy chain antibody, single domain antibody, a VH domain, or a fragment thereof, wherein amino acids at positions indicated are substituted by amino acids indicated, said positions determined according to the Kabat numbering.

EXAMPLES

Example 1: Camelization of isolated VH1

Classically, a recombinant VH domain is isolated from scFv libraries. Such VH domains usually originate from a cloning artifact for example by the cloning of VH instead of VH-VL, or they may originate from a gene recombination within the clone, due for example to instability of the linker sequences, resulting in the deletion of the VL gene fragment. These molecules are normally difficult to work with because of their low expression yields in, for example, bacterial and other expression systems and their low solubility. The inventors show that these VH molecules may be better expressed and show a higher solubility by introducing a mutation changing the Trp on position 103 into Arg. This is a much easier and more straightforward mutation than Val37Phe, Gly44Glu, Leu45Arg and Trp47Gly or part of it as originally carried out by Davies and Riechmann (1994). It has the additional advantage that it could be performed on all VH sequences, not only of human origin, but of all other species having antibodies with an Ig-fold.

Example 2: Generation of soluble single domain from a scFv antigen binding fragment.

In cases where a minimal size of the antigen binding fragment is envisaged, it might be an advantage to design a single domain from an existing scFv. The VH domain has specific interest since this domain, in principle, provides specificity and is the largest contributor to affinity. Single domains have further advantages due to their smaller size. Although it has been repeatedly shown that VH domains retain sufficient activity to interact with antigen, VH domains are known to be sticky and insoluble. The present invention shows that these problems can be remedied by the Trp103Arg substitution.

Example 3: Effect of Arg103Trp mutation on solubility and antigen binding of VHH.

3.1 Production and purification of wild type and mutant VHH.

The gene fragment encoding the anti-β-lactamase VHH TEM04 was mutagenized by PCR using the FR4 specific primer A4short-TEM04 (5'-GGA GAC GGT GAC CTG GGT CCC CTG GCC CCA TAC GAC-3'), thereby changing the wild type residue Arg on position 103 to Trp103. Using a similar approach, the anti-carcinoembryonic antigen (CEA) VHH CEA71, was mutated with primer A4short-CEAVH (5'-GGA GAC GGT GAC CTG GGT CCC CTG GCC CCA GGG GC-3').

The *E. coli* production vector pHEN6 was used for expression of the wild type and mutated VHH fragments. pHEN6 is derived from pHEN1 (Hoogenboom et al. (1991)), pHEN6 encoding the hexahistidine tag sequence for purification of VHH and lacking the phage M13 gene3. The PCR-products and vector were digested with *Nco*I-*Bst*EII and loaded on a 1% agarose gel. Fragments and vector were purified from gel with Jetsorb, ligated, transformed into WK6 competent cells and plated onto LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Mutation of R to W on position 103 was confirmed by sequencing (Fig. 1).

For each construct pre-cultures were started in triplicate in 10 ml of LB-medium containing 100 µg/ml ampicillin and 2% glucose. 330 ml cultures (in TB-medium with 100 µg/ml ampicillin) were inoculated with 3 ml of preculture and grown at 37°C. Cultures were induced at OD600nm= 0.4 with 1 mM IPTG and grown overnight at 28°C. No significant differences were observed in cell densities after induction between the wild type and mutant (VHH TEM04: OD600(wt) = 1.05 ± 0.25; OD600(mut) = 1.25 ± 0.05 ; VHH CEA71: OD600(wt) = 1.48 ± 0.28; OD600(mut) = 1.30 ± 0.20), suggesting that no toxic products were expressed.

The cells were boiled in reducing sample buffer and loaded on 15% PAGE, normalized for the number of cells (OD600nm= 0.1). The proteins were blotted on nitrocellulose and blocked overnight in PBS containing 1% casein. The hexahistidine-tagged VHH was detected with mouse anti-Histidine monoclonal

antibody (Serotec, diluted 1:1000 in PBS) and after 4 washes with PBS-0.5% tween-20 incubated with anti-mouse alkaline phosphatase conjugate (Sigma, diluted 1:1000) using NBT and BCIP as chromogenic substrates. It can be concluded that the VHH TEM04 mutant is expressed at much lower levels than its wild type derivative, while for VHH CEA71 no differences were observed (Fig. 2).

Periplasmic extracts were made from all cultures by resuspending the cells in 4 ml TES (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose; pH 8.0). The suspension was incubated for 30 minutes on ice. Subsequently 6 ml 0.25xTES was added and the incubation on ice was continued for 20 minutes. Periplasts were removed by centrifugation for 20 minutes at 10,000 rpm and 4°C in SS34-rotor (Sorval). VHH was purified by IMAC using Ni-NTA (QIAGEN). The yields were determined by measuring the OD280nm using the calculated molar extinction coefficients (VHH TEM04 E(R103) = 2.168 and E(W103)= 2.582; VHH CEA71 E(R103) = 1.444 and E(W103)= 1.865) (Table 1). As was observed by Western blot analysis the yield of mutant antibody for VHH TEM04 was much lower than for its wild type, while for VHH CEA71 no difference was found. The purified VHH were analyzed on a coomassie stained PAGE (Fig. 3), which revealed that β-lactamase was co-purified as a complex with VHH TEM04, both for the wild type and the mutant form.

VHH	Yield (mg/l)	
	R (wild type)	W (mutant)
TEM04	14 ± 5	2.3 ± 0.7
CEA71	33 ± 4	32 ± 2

Table 1: Production yields of wild type (R on position 203) and mutant (W) VHH expressed per liter of culture.

3.2 Antigen binding characteristics of VHH CEA71 variants.

An ELISA was performed to compare the antigen binding characteristics of the wild type and the mutant VHH CEA71. A microtiter plate (Maxisorp, NUNC) was coated overnight at 4°C with CEA (Scrips) at a concentration of 1 µg/ml (in PBS), and blocked for two hours at room temperature (RT) with 300 µl 1% casein in PBS. The plates were washed three times with PBS-tween. Dilution series (10 µg/ml to 4.57 ng/ml, dilution factor three) of all purified samples were incubated in triplicate (100 µl/well) for 2 hours at RT. Plates were washed six times with PBS-tween, after which binding of VHH was detected by incubation with mouse anti-Histidine mAB (Serotec; 1:1000 diluted; 100 µl/well) for 1 hour at RT followed by anti-mouse-alkaline phosphatase conjugate (Sigma, 1:2000 diluted), also for 1 hour at RT. Staining was performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in 1M diethanolamine, 1mM Mg_2SO_4, pH9.8) and the signals were measured after 30 minutes at 405 nm. The CEA wild type VHH still binds at approximately tenfold lower concentrations than the mutant form (Fig. 4). This means that either 90% of the mutant protein is not correctly folded (thus inactive) or that the affinity of the mutated VHH is tenfold lower.

For VHH TEM04 no ELISA was performed, but on the coomassie stained gel (Fig. 3) the co-purified β-lactamase seems to have a similar intensity as the VHH, suggesting that the R103W mutant is produced completely in an active form. It therefore can be assumed that the introduction of Tryptophan on position 103 decreases the affinity.

3.3 Solubility of VHH CEA71 wild type and R103W mutant.

The solubility characteristics were examined by determination of the concentration of ammonium sulfate, at which the VHH starts to precipitate. Therefore a saturated stock solution of ammonium sulfate was prepared by dissolving an excess of salt in a limited volume of water. After equilibration for 2 hours at RT, the solid particles were removed by centrifugation at 4300 rpm for 10 minutes and the supernatant

(100% ammonium sulfate solution) was used to make dilutions of 0-80%. 60 µl sample was added to 300 µl ammonium sulphate solution and mixed for 18 hours at 4°C. This mixture was centrifuged for 10 minutes at 13000 rpm in an Eppendorf centrifuge. The amount of soluble VHH in the supernatant was determined in ELISA deduced from the degree of antigen binding (Fig. 5A) and on the other hand by measuring the protein concentration with OD280 (Fig. 5B).

From both types of measurements it can be concluded that the wild type anti-CEA VHH CEA71 started to precipitate at an ammonium sulfate concentration of 65%, while the mutant form shows signs of precipitation at 58%. This experiment clearly demonstrates that the introduction of Tryptophan on position 103 in the context of the wild type sequence of VHH CEA71, which has Arginine on this position, decreases its solubility.

Example 4: Cloning, selection and production of functional single domain heavy chain antibody fragments.

Dromedaries and llamas were immunized intramuscularly with a cocktail of antigens using Freund's complete adjuvant (first injection) and Freund's incomplete adjuvant (subsequent injections). Dromedaries were immunized with CEA (Carcino Embryonic Antigen), ovalbumine (OVA), PSA (Prostate Specific Antigen), Variant Surface Glycoprotein trypanosome (VSG), ß-lactamase, carbonic anhydrase, Cutinase, Potyvirus and Lysozyme. Llamas were immunized with Poly A Binding Protein Type 2 (PABP2), Linoic Acid BSA-conjugate, a humanized mouse mAb to CD40 (Hu-anti-CD40), human serum albumin (HSA), *Salmonella typhimurium*, Rotavirus. Following 6 injections with a one-week interval, a blood sample of 100 ml was collected. PBL cells were separated on a Ficoll-Paque Plus gradient (Amersham Biosciences). Total RNA was isolated from these cells using an acid guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987) and cDNA was prepared using M-MLV RT (Gibco BRL) and random oligonucleotide primers (Amersham

Biosciences). With the primers Call001 (5'-GTCCTGGCTGCTCTTCTACAAG-3') and Call002 (5'-GGTACGTGCTGTTGAACTGTTCC-3'), annealing to the leader sequence and the CH2 exon of all camelid immunoglobulins respectively, the gene fragments coding for the variable domain were amplified by PCR. To introduce a *NcoI* restriction site, the gene fragments were reamplified using an equimolar mixture of upstream primers SM017 (5'-CCAGCCGGCCATGGCTGATGTGCAGCTGGTGGAGTCTGG-3', and SM018 (5'-CCAGCCGGCCATGGCTCAGGTGCAGCTGGTGGAGTCTGG-3') in combination with Call002 in a nested PCR. In the final amplification the A4short primer (5'-CATGCCATGACTCGCGGCCCAGCCGGCCATGGC-3') was used on one hand to introduce a *Sfi*I site and on the other hand the primers as described before were used to introduce the key residues on position 103 and a *Not*I site: primer 1 (R103), primer 2 (R103), primer 3 (K103), primer 4 (Q103), primer 5 (L103), primer 6 (F103), primer 7 (G103), primer 8 (S103), primer 9 (P103), primer 10 (Y103). The PCR products were cloned using the *Sfi*I/*Not*I restriction enzymes into the phagemid vector pHEN4 (a derivative of pHEN1 (Hoogenboom et al., 1991) with a HA-tag downstream the cloning sites of the heavy chain antibody fragment). The repertoire was expressed on phage following infection with M13K07 helper phages. Specific binders were selected using the principle of phage display and panning (Ghahroudi et al., 1997).

Single domain heavy chain antibodies specific for CEA (CEA1 and CEA72), PSA (N3-A, N8-B, C9-B, C11-B, C12-A, C1-B, C24-A, N13-A, N15-B), HSA (ALB1, ALB2, ALB3, ALB4, ALB5), Hu anti-CD40 (CD40-1, CD40-2, CD40-3, CD40-4, CD40-5, CD40-6, CD40-7), OVA (B13, 1DBOVA11, 1DBOVA23, 1DBOVA43, A2-19, A4-17, B368, R24), VSG (cAbAn04), ß-lactamase (cAbBLA01, cAbTEM04), carbonic anhydrase (1D2CA30), PABP2 (C4 PABP2, E3 PABP2, F6 PABP2), *Salmonella typhimurium* (MPOD6 salmon), Rotavirus (1-F6 RTV), Linoic Acid (LA-1), Cutinase

(CutIII19, A4cut9, CACU13, CABCUT4, CU16), Potyvirus (48dpvy, 348DPVY, 1648DPVY,1048DPVY23, PVYIA15, PVYIA2, PVYIA1, PVY17) and Lysozyme (1D2L28) were isolated and evaluated for expression, binding in ELISA and affinities. The sequences are listed below; amino acid position 103, as determined by the Kabat numbering is indicated in bold,

N3-A
DVQLQESGGSLVQPGGSLRLSCAASGFTFSAYYMIWVRQAPGKGLEWVSGISANGRDTLYEDSVEGRFAISRDNA
KNTLYLQMNSLRSEDTALYYCVIGALITGR**R**GQGTQVTVSS

N8-B
DVQLQESGGGLVQPGGSLRLSCAASGFLFSDTYMTWARQAPGKGLEWLGGISKDGSGTLYEDSVEGRFTISRDNA
KNTLYLQMNSLKSEDTALYYCSTGALLPTRP**Q**GQGTQVTVSS

C9-B
DVQLQESGGGLVQPGGSLRLSCAASGFTFSNHYMTWVRQAPGKGLEWVSVISNDGRYTDYADSVKGRFTISRDNA
KNTLYLQMNSLKTEDTALYTCVRGYYLTNLPAGD**R**GQGTQVTVSS

C11-B
DVQLQESGGGLVQPGGSLRLSCAASGFIFSNTYMTWVRQAPGKGLEWVSGISADGRDTLYADSVEGRFAISRDNA
KNTLYLQMNSLRSEDTALYYCVTGALMTGR**R**GQGTQVTVSS

C12-A
DVQLQESGGGLVRPGGSLRLSCAASGFLFSGTYMTWARQAPGKGLEWLCGINKDGSGTLYADSVEGRFTCSRDNA
KNTLYLQMNSLKSEDTALYYCSTGALLPTRP**Q**GQGTQVTVSS

C1-B
DVQLQESGGGLVQPGGSLRLSCAASGFTFSTSYMTWARQAPGKGLEWVSGINRDGNNPLYADSVEGRFTVSRDNA
KNTLYLQMNSLKSEDTALYYCVAGALVAGA**R**GQGTQVTVSS

C24-A
DVQLQESGGGLVQPGGSLRLSCAASGFAFTPSYMSWVRQAPGKGLEWVSVISNDGRYTDYADSVKGRFTISRDNA
KNTLYLQMNSLKTEDTALYTCVRGYYLTNLPAGD**R**GQGTQVTVSS

N13-A
DVQLQESGGGSVQPGGSLRLSCAASGFTFKDASMNWVRQAPGKGLEWVSAINGGGTVTDYADPMEGRFTISRDNA
KNTLYLQMNSLNFEDTALYYCATGWLFRANNY**R**GQGTQVTVSS

N15-B
DVQLQESGGGSVQAGGSLRLACAATAYTYDSNVLGWFRQAPGKEHEGVAVIYTGTRTTYYADSVKGRFTISQDNA
KNTVYLQMNSLKPGDTAMYFCAANVRLGGVWSFDY**R**GQGTQVTVSS

ALB-1
QVQLQESGGGLVQPGGSLRLSCAASGFAFSSFPMTWVRQAPGKGLEWVSGILEGGGSPAYADSVKGRFTISRDDA
KNTLYLQMNSLKPEDTAVYYCAKGYVYAREGA**R**SQGTQVTVSS

ALB-2
QVQLQESGGGLVQPGGSLRLTCTASGFAFSNFGMSWVRQPPGKGLEWVSAISADSSTKNYADSVKGRFTISRDNT
KKMLYLEMNSLKPEDTAVYHCVIGRGSA**S**SQGTQVTVSS

ALB-3
QVQLQESGGGLVQPGNSLRLSCAASGFAFGNFGMSWVRQAPGKEPEWVSSIDSIGSDTLYADFVKGRFTISRDNA
KSTLYLQMNSLKPEDTAVYYCTIGGSLSR**S**SQGTQVTVSS

ALB-4
QVQLQESGGGLVQPGNSLRLSCAASGFSFRSFGMSWVRQAPGKGPEWVSSINSSGDDTRYTDSVKGRFTISRDNA
KSTLYLQMNSLKPEDTAVYYCTIGSSISR**S**SQGTQVTVSS

ALB-5
QVQLQESGGGLVQPGGSLRLTCTASGFAFSSFGMSWVRQPPGKGLEWVSAISADSSTKNYADSVKGRFTISRDND
KKMLYLEMNKLKPEDTAVYHCVIGRGSP**S**SQGTQVTVSS

CEA1

QVQLVESGGGLVQPGGSLRLSCAASGFTFSKYDMSWVRQAPGKGLEWVSRISSGGGSTYYADSVKGRFTISRDNA
KNTLYLQMNSLKPEDTAVYYCATPTYSSDYRGLP**P**GQGTQVTVSS

CEA72
QVQLVESGGGLVQPGGSLRLSCAASEFTFSSSYMSWVRQAPGKGLEWVSGINTDGSFTRYADSVKGRFTISRDNA
KNTLYLQMNSLKSEDTALYYCAVGGGLGYGP**R**GQGTQVTVSS

B13
QVQLQASGGGLVQPGGSLRLSCAASGFDFMNVYMTWVRQAPGKGVEWVSGISVSGSITHYSESVKGRFTISRDNA
KNMLYLQMNSLKSEDTARYYCARGGYNRYYGA**L**GQGTLVTVSS

1DBOVA11
QVQLVESGGGSVQ?GESLRLSCVASGFTFDV?YMNWVRQAPGKGLEWVSGISASGY?TTYA??VKGRFTISRDNA
KNTLYLQMNSL??T**R**GQGTQVTVSS

1DBOVA23
QVQLVESGGGSVQAGGSLIISCAASGFDFSNNYMTWVRQAPGKGVEWVSGISVSGSITHYTDSVKGRFTISRDNA
KNTLYLQMNSLRSEDTARYYCGTGGYGRYYGT**L**GQGTQVTVSS

1DBOVA43
QVQLVESGGGLVQPGGSLRLSCVGSGFTFSSYYISWVRQAPGKGLEWVSGISGSGATTSYTDSVKGRFTISRDNA
KNTVYLQLNSLETEDSAMYYCRLGYGTPPGGVWPS**Q**RQGTQVTVSS

A2-19
QVQLQASGGGLVQPGGSLKLSCVVSGFLFSNYAFSWVRQAPGKGLEWVSTIGTSSGYTNYAPSVKGRFTISRDNA
KNTVYLQLNSLKTEDTAMYYCRRPGTDE**R**GQGTQVTVSS

A4-17
QVQLQASGGGLVQPGGSLRLSCAASGFDFSNVYMTWVRQAPGKGVEWVSGISVSGSITHYSDSVKDRFTISRDNA
KNTLYLQMNSLKSEDTARYYCARGGYNTYSGA**L**GQGTQVTVSS

B368
VQLVESGGGSVQAGGSLILSCTASGLPYKSYCMGWFRQAAGKEPEGVATINSGTGSKFYTDSVKGRFTISLDNDN
NRVYLEMSSLKPEDTATYYCAAGQRHSCGYVLKNTDGWTH**R**AQGTQVTVSS

R24
SAQVQLQASGGGLVQPGGSLKLSCVVSGFLFSNYAFSWVRQAPGKGLEWVSTIGTSSGYTNYAPSVKGRFTISRD
NAKNTVYLQLNSLKTEDTAMYYCRRPGTDE**R**GQGTQVTVSS

cAbAn04
QVQLVESGGGSVEAGGSLRLSCVVSGYSVSIGCMAWFRQAPGSGREGVAGISRGGSMTDYTASVKGRFTISRDND
QRTVTLQMNSLKPEDTAVYYCARDGPEIATMIGGS**R**GRGTQVTVSS

cAbBLA01
QLQLVESGGGSVQSGGSLRLSCKVSGYIGSTNCMGWFRQAPGKEREGVASLFTGSGNTYYGDSVKGRFTISEDNA
KNTVSLQMNSLKPEDTAMYYCASSSNVGSDESCGRKNTRQFVYTY**Q**GQGTQVTVSS

cAbTEM04
QVQLVESGGGLVQAGGSLRLSCAASGFTFSSAWMTWVRQAPGKGLEWVTSIATDGSTDYADSVKGRFTISRDNAK
NTLYLQLNSLNTEDTAVYYCAKDRWGYVV**R**GQGTQVTVSS

1D2CA30
QVQLVESGGGSVQAGGSLRLSCAASGYTVSTYCMGWFRQAPGKEREGVATILGGSTYYGDSVKGRFTISQDNAKN
TVYLQMNSLKPEDTAIYYCAGSTVASTGWCSRLRPYDYHY**R**GQGTQVTVSS

C4 PABP2
QVQLQESGGGLVQPGGSLRLSCAASGFTFSRSWMYWVRQAPGKGLEWVSSITPGGSEPFYVDSVKGRFTISRDNA
KNTLYLQMNSLKSEDTAVYFCAKDSKNGP**R**GQGTQVTVSS

E3 PABP2
QVQLQESGGGLVQPGGSLRLSCAASGFTFSRSWMYWVRQAPGKGLEWVSSITPGGTEAFYADSVKGRFTISRDNA
KNTLYLQMNSLKSEDTALYFCAKDSKNGP**R**GQGTQVTVSS

F6 PABP2
QVQLQESGGGLVQPGGSLRLSCATSGFIFSDYWMYWVRQAPGKGLEWVSSITPGASTTLYADSVKGRFTISRDNA
KNTLYLQMNSLKSEDTAVYYCAKGSKIGP**R**GQGTQVTVSS

LA-1
QVQLQDDSGGGLVQPGGSLKLSCAASGFTFSNYEMSWVRQAPGKGLEWVSSINNGGDITYYANSVKGRFTISRDN
TKNTLYLQMNSLKSEDTAVYYCKVPNRRL**R**GPGTQVTVSS

CD40-1

QVQLVESGGGLVQPGGSLRLSCAASGFAFSRYSMYWVRQAPGKGLEWVSEIYPDGNGWYTSSVKGRFTISRDNDK
NMLYLQMNSLKPDDTAVYYCALSRSGQG**R**GQGTRVTVSS

CD40-2
EVQLVESGGGLVQAGGSLELSCSFGGRAFDRYFMAWFRQAPGKGLEWVSRIYSGGSTSYADSVKGRFTISRDNAK
NTLYLQMNNLKPEDTAVYYCDIAGR**R**GQGIQVTVSS

CD40-3
EVQLVESGGGLVQAGDSLRLSCAASGRTFNTVDMGWFRQAPGKEREFVAHISWRGGSTYYADSVKGRFTISRDNA
KNTLYLQMNNLKPEDTAVYYCDIAGR**R**GQGTQVTVSS

CD40-4
QVQLVESGGGLVQPGGSLRLSCAASGFAFSRYSMYWVRQAPGKGLEWVSEIYPDGNGWYTSSVKGRFTISRDNDK
NMLYLQMNSLKPDDTAVYYCALSRSGQG**R**GQGTRVTVSS

CD40-5
EVQLVESGGGLVQAGGSLELSCSFGGRAFDRYFMAWFRQAPGKGLEWVSRIYSGGSTSYADSVKGRFTISRDNAK
NTLYLQMNNLKPEDTAVYYCDIAGR**R**GQGIQVTVSS

CD40-6
EVQLVESGGGLVQAGDSLRLSCAASGRTFNTVDMGWFRQAPGKEREFVAHISWRGGSTYYADSVKGRFTISRDNA
KNTLYLQMNNLKPEDTAVYYCDIAGR**R**GQGTQVTVSS

CD40-7
AVQLEESGGDSVQAGGSLRLSCAASGFTFSRYSMYWVRQPPGKGLEWVSEIYPDGNGWYTSSVKGRFTISRDNDK
NMLYLQMNSLKPDDTAVYYCALSRSGQG**R**GQGTRVTVSS

MPOD6 Salmon
QVQLQESGGGLVQPGGSLRLSCAASGFTFNDYFMNWVRQAPGKGLEWVSGIYSDGSKTYYGDSVKGRFTISRDNA
KNTLYLQMNSLKSEDSAVYYCTRGTGWSSTPYTY**R**GQGTQVTVSS

1-F6 RTV
QVQLQEVRGRLVQLGGSLRLSCAASGFTFKYYAMSWVRQAPGKGLEWVSYINDNGGYTDYSDSVKGRFTISRDNA
KNTLYLHMNRLKPEDTAVYFCAKWDTDAVSSSRYKTHNGDI**R**GPGTQVTVSS

CUTIII19
QVQLVESGGGLVQAGESLTLSCTASGGSFNNWHMGWFRQAPGTEREFVAAIRRAYGSTFYADSVKGRFTI
ARDNAKNTVYLQMSSLKPEDSAVYYCAAKRAFRVGGDFEY**Y**GQGTQVTVSS

A4cut9
QVQLQASGGGLVQPGGSLRLSCAASGFTFSTYYMNWVRQAPGKGLEWVPGINKDGSVSHYADSVKGRFTISRDNA
KNTLYLRMNSLKSEDTALYYCATIAGFRVGG**G**PGGTQVTVSS

CACU13
DVQLVESGGGLVQPGGSLRLSCAASGFRFDSVAMTWVRQTPGKGLEWVSSISWDGTTTSYAASVKGRFTISRDNA
KNTLYLQLDSLKTEDTAMYYCTKTGVDYRDSRD**R**GRGTQVTVSS

CABCUT4
QVQLVESGGGLVQPGGSLRLSCAASGFRFDSVAMTWVRQAPGKGLEWVSSISWDGTTTSYAASVKGRFTISRDNA
KNTLYLQLDSLNTEDTAMYYCTKTGVDYRDSRS**R**GQGTQVTVSS

CU16
QVQLVESGGGSVQAGGSLKLTCELSGFNGRSNCMGWFRQVLGKDREGVAAINHPEGSEFYDDSVKGRFKITRDGL
KDADSLQMNNLKPEDTATYYCALRPYDCYSGAWSPADFYYR**G**ARGTQVTVSS

48dpvy3
QVQLQASGGGSVEAGGSLRLSCAASGDTAKLNCMAWFRQAPGKERERVASLSTRLTTTSYTDSVKGRFTISQDTA
TNTVYLEMNSLQPEDTAVYYCQLSRGGTNY**R**GQGTLVTVSS

48DPVY16
QVQLQASGGGSVQAGGSLRLSCAASGYTYSSNCMGWFRQALGKEREGVAAIYTGGGSTYYADSVKGRFTISQDNA
KNTVYLQMNSLKPEDTAMYYCAASLLPLVAGIGVWDAFDY**R**GQGTQVTVSS

48DPVY10
QVQLQASGGGSVQAGGSLRLSCVASQYEYSNNYIAWFRQAPGKEREGVAAIYTGGVTRASPYYADPVKGRFSISK
DNAKNTVYLQMNDLKPEDSGTYICASSIHGLGNPLRSEFSY**Y**GQGTLVTVSS

48DPVY23
QVQLQASGGGSVEAGGSLRLSCAASGDTAKLNCMAWFRQAPGKERERVALLSTRLTTTSYTDSVKGRFTISQDTA
TNTVYLEMNSLQPEDTAIYYCAARWAGRSCLVSVYDY**Y**GQGTLVTVSS

PVYIA15

QVQLVESGGGLVQPGGSLRLSCAASGFTFSNYAMSWVRQAPGKGLEWVSGIKSGGGRTYYADSVKGRFTISRDNA
KNTLTLQLNSLKTEDTAMYYCAKGARYDSDYDVTWLDSY**S**GQGTQVTVSS

PVYIA2

DVQLVESGGGSVQAGGSLRLSCTASGLRLNTYHMSWVRQAPGKGLEWVSTIYIGGTTTSHANSVSGRFTISRDDA
KNTLYLQMNNLKPEDTAVYFCATGSVNAYGV**K**GQGTQVTVSS

PVYIA1

QVQLVESGGGSVQAGGSLTLSCTVSGYDFNRCSMNWYRENPGKEREFVAGIDSDGTTTYADSVKGRFTISHDNTR
NTLYLQMNSLKSEDTALYYCRLGGLRTWPQYGY**R**GQGTQVTVSS

PVY17

QVQLVESGGGLVQPGGSLRLSCTASGLRLNTYHMSWVRQAPGKGLEWVSTIYIGGTTTSHANSVSGRFTI
SRDDAKNTLYLQMNNLKPEDTAVYFCATGSVNAYGV**K**GQGTLVTVSSAA

1D2L28

QVQLVESGGGLVQPGGSLRLSCAASGFAFSIYRMSWVRQAPGQGLEWVSSIDSGGGITYYADSVKGRFTI
SRDNAKNTLYLQLNSLKTEDTAMYYCARGHYLYDDDIFTGA**K**GQGTQVTVSSGR

For each construct 10 ml pre-cultures were started in TB containing 100 µg/ml ampicillin and 2% glucose. For each pre-culture, 4 x 330 ml culture was started at 37°C in TB containing 100 µg/ml ampicillin with 3 ml of the overnight culture. Cultures were induced with 1 mM IPTG at OD600nm= 0.4 and grown overnight at 28°C.

Periplasmic extract was prepared for all overnight cultures. The overnight cultures were centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was removed and the pellet was re-suspended in 16 ml TES (0.2 M Tris-HCl, pH= 8.0, 0.5 mM EDTA and 0.5 M sucrose). This mixture was incubated for 30 minutes on ice. 24 ml 0.25xTES was added, incubated on ice for 20 minutes and centrifuged for 20 minutes at 10,000 rpm. The supernatant was purified on Ni-NTA (QIAGEN), and dialyzed overnight against PBS. OD280 was measured and the yield (in mg) of purified material per liter of culture was determined. Kd's were determined on BIAcore are given in the table below.

Name of binder	Antigen recognized by antibody	Kd (nM)	Expression level (mg/l culture, purified material)	Host
N3-A	Prostate specific antigen	-	0.25	Dromedary
N8-B	Prostate specific antigen	1.6	6.2	Dromedary
C9-B	Prostate specific antigen	3.9	2.1	Dromedary

C11-B	Prostate specific antigen	2.6	6.1	Dromedary
C12-A	Prostate specific antigen	2.6	1.2	Dromedary
C1-B	Prostate specific antigen	0.8	0.75	Dromedary
C24-A	Prostate specific antigen	-	5.0	Dromedary
N13-A	Prostate specific antigen	-	0.25	Dromedary
N15-B	Prostate specific antigen	-	0.65	Dromedary
CEA1	Carcino Embryonic Antigen			Dromedary
CEA72	Carcino Embryonic Antigen		8.4	Dromedary
B13	ovalbumin		<13	Dromedary
1DBOVA11	ovalbumin			Dromedary
1DBOVA23	ovalbumin			Dromedary
1DBOVA43	ovalbumin			Dromedary
A2-19	ovalbumin			Dromedary
A4-17	ovalbumin			Dromedary
B368	ovalbumin			Dromedary
R24	ovalbumin			Dromedary
cAbAn04	Variant surface glycoprotein trypanosome			Dromedary
cAbBLA01	ß-lactamase	<1	0.4	Dromedary
cAbTEM04	TEM1			Dromedary
1D2CA30	Carbonic anhydrase			Dromedary
A4Cut9	Cutinase			Dromedary
CACU13	Cutinase			Dromedary
CABCUT4	Cutinase			Dromedary
CU16	Cutinase			Dromedary
48dpvy	Potyvirus			Dromedary
348DPVY	Potyvirus			Dromedary
1648DPVY	Potyvirus			Dromedary
1048DPVY23	Potyvirus			Dromedary
PVYIA15	Potyvirus			Dromedary
PVYIA2	Potyvirus			Dromedary
PVYIA1	Potyvirus			Dromedary
PVY17	Potyvirus			Dromedary
1D2L28	Lysozyme			Dromedary
LA-1	Linoic acid			Llama
C4 PABP2	Poly A Binding Protein Type 2			Llama
E3 PABP2	Poly A Binding Protein Type 2			Llama
F6 PABP2	Poly A Binding Protein Type 2			Llama
CD40-1	humanised mouse mAb to CD40	2	8	Llama
CD40-2	humanised mouse mAb to CD40			Llama

CD40-3	humanised mouse mAb to CD40			Llama
CD40-4	humanised mouse mAb to CD40			Llama
CD40-5	humanised mouse mAb to CD40			Llama
CD40-6	humanised mouse mAb to CD40			Llama
CD40-7	humanised mouse mAb to CD40			Llama
MPOD6 salmon	Salmonella Typhimurium			Llama
1-F6 RTV	Rotavirus			Llama
CutIII19	Cutinase			Llama
ALB-1	Human serum albumin		15	Llama
ALB-2	Human serum albumin		15	Llama
ALB-3	Human serum albumin		15	Llama
ALB-4	Human serum albumin		15	Llama
ALB-5	Human serum albumin		15	Llama

Alignment of the CEA1 binder and a human VH3 germline (DP-47) revealed a high degree of homology (two amino acid changes in FR1 on position 1 and 5 and four changes in FR3 on position 74, 83, 84 and 94), as shown below:

```
DP-47  EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVSAISGSGGSTYY
CEA1   QVQLVESGGGLVQPGGSLRLSCAASGFTFS KYDMS WVRQAPGKGLEWVSRISSGGGSTYY

DP-47  ADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK ----------------------
CEA1   ADSVKG RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAT PTYSSDYRGLPPGQGTQVTVSS
```

A specific binder for the CEA tumor antigen, with high homology to the human germline gene DP-47 was therefore an ideal candidate to further humanize and evaluate the influence of mutagenesis on binding affinity in ELISA and BIAcore.

Mutagenesis of the residues in FR1 had no significant influence on specificity, binding affinity and expression level. Mutagenesis of most of the FR3 residues did

not result in loss of specificity, affinity and expression levels. The most humanized and best performing CEA1 mutant was chosen for immunogenicity studies. Baboons were immunized intravenously, intramuscularly and subcutaneously with a weekly dose of 1 mg/kg body weight. Blood samples were taken and humoral response was evaluated in ELISA. No significant antibody response was raised against the CEA1 mutant.

Example 5: Construction of a functional single domain heavy chain antibody fragment library derived from human and mouse VH.

cDNA templates were made starting from blood samples from 20 non-immunized human donors (or from other species containing antigen binding molecules with an Ig-fold). The peripheral blood lymphocytes (PBL) were isolated on Ficoll-Paque gradients (Amersham Biosciences). Total RNA was prepared individually from the 20 samples of PBL's as described in example 4. First strand cDNA was also individually synthesized from total RNA with random hexamers as primers (as in example 4). Mutagenesis was carried out using a Framework 1 specific primer that is 5' linked to a SfiI site, as backward primer, and the mutagenic primer:

3'- ACC CGA GGA GCC TGG GAC CAG TGG CAG – 5'

or

3'- ACC CCG GTG AGG AGG GTC CAG TGG CAG – 5'

as forward primer. The DNA obtained was cut with *Bse*RI and ligated to the double stranded primer:

5'- pC GTC AGG GGC CAA GGA ACC CAG GTC ACC GTC TCC TCA -3'

3'- CTG CAG TCC CCG GTC CCC TGG GTC CAG TGG CAG AGG AGT -5'

for the PCR product obtained with the first mutagenic primer, or

5'- pAGG GGC CAA GGA ACC CAG GTC ACC GTC TCC TCA – 3'

3'- GT TCC CCG GTC CCC TGG GTC CAG TGG CAG AGG AGT -5'

for the PCR product obtained with the second mutagenic primer.

This primer restores the CDR3 and Framework 4 coding regions. The resulting DNA was amplified using the same forward primer as above, and using

3'- C TGG GTC CAG TGG CAG AGG AGT CGCCGGCG -5'

as backward primer. This primer created a *Not*I site at the end of the VH coding sequence. The resulting material was cut with *Sfi*I and *Not*I and the resulting fragment cloned in pHEN4 to yield a phage display library.

The library was selected with a panel of antigens, such as Human Serum Albumin and human IgG1, thereby obtaining large numbers of antigen specific antibody fragments. Sequence analysis revealed the introduced residue on position 103. The produced and His6-tagged VHH fragments showed good solubility characteristics, good specificity (not reactive against irrelevant antigens) and high affinities (range of 2 to 50 nM).

A mouse was immunized with a set of antigens (CEA and PSA) and after four weeks the spleen was removed, homogenized in guanidinium thiocyanate buffer with a Polytron homogenizer, debris removed by low speed centrifugation and total RNA extracted using the method described before. As was described above the mouse VH encoding gene segments were amplified thereby introducing the variant residues on position 103. Upon selection high affinity VH fragments were selected with good characteristics with respect to solubility.

Example 6: Isolation, sequencing and production of human TNF specific fragments.

Selection of binders for tumor necrosis factor alpha (TNF) from a phage library was carried out as described in Example 4. After panning the library, 48 individual clones were selected and tested in a phage ELISA on immobilized TNF and BSA.

The clones for which the signal on TNF was superior to the one obtained on BSA were selected for further characterization. By sequencing the selected clones, we were able to eliminate identical clones and to demonstrate that W103 was no longer encoded in the selected fragments.

The gene segments encoding the selected antibody fragment were recloned in the pHEN6 expression vector as described in example 3, which allowed us to produce the recombinant antibody fragment as soluble periplasmic proteins. The recombinant antibody fragments were purified to homogeneity from the periplasmic fraction by IMAC on Ni-NTA agarose and subsequent gel-fitration chromatography on Superdex-75. The purity was determined by SDS-PAGE.

6.1 Applications of anti-TNF antibody fragments.

6.1.1 Therapeutic applications.

Anti-TNF specific fragments were tested in L929 murine fibrosarcoma cells following the protocol as described by Ameloot et al. (2001). L929 cells were seeded in 96-wells mirotiter plates at 30,000 cells per wells. The next day, purified recombinant antibody fragments were added to some wells, whereas only PBS was added to control wells. In this experiment the final concentration of antibody fragment was 1 micromolar.

Subsequently a lethal dose of TNF was added to those wells where antibody was added and also to part of the control wells. After 18 hours, the level of surviving cells was estimated by the colorimetric method. In this way we demonstrated that these antibody fragments have the ability to neutralize the cytotoxic effect of TNF and have therapeutic potential.

6.1.2 Diagnostic application of anti-TNF VHH in ELISA.

To individual wells of a microtiter plate, we added 100 µl of the antibody fragments at a concentration of 5 µg/ml in PBS. After incubation overnight at 4°C the plate is blocked with 1% BSA in PBS. The presence of functional immobilized antibody fragment was demonstrated by the binding of biotinylated TNF at 1 µg /ml. The

presence of bound biotinylated TNF was demonstrated with streptavidin-alkaline phosphatase conjugate and subsequent reaction with para-nitrophenyl-phosphate.

6.1.3 Application of anti-TNF VHH in antibody arrays.

Two different formats of antibodies arrays were tested, i.e. on nitrocellulose filters and glass slides. For the filter method 2 µl of the purified antibody fragments at concentration of 1mg/ml in PBS were spotted with a micropipette on a nitrocellulose sheet. After drying, the sheet was blocked with 1% BSA in PBS. The presence of functional immobilized antibody fragment on the nitrocellulose sheet was demonstrated with biotinylated TNF. The presence of bound TNF was demonstrated with streptavidin-alkaline phosphatase conjugate and NBT-BCIP reagent. The appearance of dark spots on those positions where the TNF-specific fragments were applied proved that these antibody fragments retain functionality when passively coated on a solid support. This approach can be used for random screening and selection of antigen-specific fragments.

For the glass slide type of antibody array the anti-TNF antibody fragments were covalently immobilized. Purified antibody fragments were diluted to a concentration of 200 µg/ml in PBS containing 20% glycerol. The samples where transferred to wells of a 384 well microtiter plate. Subsequently an automated contact printer was used to deliver 5 nanoliter of the antibody solutions to a commercially available glass slide (Telechem - Superaldehyde).

After application of the samples, the glass slide was incubated for 1hour in a humid chamber, to allow the reaction between the reactive aldehydes present on the glass slide and the lysine groups present on the antibody fragment surface to proceed.

The slide was subsequently blocked with 1%BSA / PBS, subsequently incubated with Cy3-modified TNF (fluorescent label Cy3-Amersham Biosciences) at 1 µg/ml and finally washed with PBS to remove unbound labeled TNF. After scanning the fluorescence intensity present on the surface of the slide, we observed an enhanced signal at those positions where the TNF specific antibody fragments were applied.

This result demonstrated that these antibody fragments were covalently immobilized with retention of binding capacity.

6.1.4 Affinity chromatography.

Purified antibody fragments at a concentration of at least 1mg/ml were dialyzed against 0.1 M sodium bicarbonate and subsequently mixed with 1 ml gel suspension (CNBr-activated Sepharose) following the protocol as described by the manufacturer (Amersham Biosciences).

After incubation for 3 hr, 100 µl of a 1M Tris pH 8 solution was added. After extensive washing, in order to remove unbound protein, the affinity resin was resuspended in 1 ml PBS.

The functionality of the immobilized anti-TNF antibody resin was tested as described below. To 1 ml of human plasma 10 µg of purified TNF was added. We then added 100 µl of the affinity resin. After of overnight incubation of this suspension, the resin was washed extensively with PBS. The pelleted beads were subsequently resuspended in 100 µl of a solution containing 1% SDS solution and boiled for 10 minutes. After centrifugation 20 µl of the supernatant was loaded on SDS-PAGE. A band of the expected molecular weight was enriched in the analyzed sample.

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Claims

1. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising, an amino acid which is neither a charged amino acid nor a C at position 45, and comprising an amino acid at position 103 independently chosen from the group consisting of R, G, K, S, Q, L, and P, and optionally an amino acid at position 108 independently chosen from the group consisting of Q, L and R, said positions determined according to the Kabat numbering.

2. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 13 (RGQGTQ) according to Figure 6, said positions determined according to the Kabat numbering.

3. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 14 (RGKGTQ) according to Figure 6, said positions determined according to the Kabat numbering.

4. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising SEQ ID NO: 15 (VXXXXXXGLXW) according to Figure 6, wherein X is any amino acid, said positions determined according to the Kabat numbering.

5. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ

ID NO: 16 (LGQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

6. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 17 (QGQGTGVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

7. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 18 (PGQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

8. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof comprising an amino acid which is neither a charged amino acid nor a C at position 45 and SEQ ID NO: 19 (SSQGTQVTVSS) according to Figure 6, said positions determined according to the Kabat numbering.

9. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1 to 10 according to Figure 6.

10. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 3, 5, 7 or 9 according to Figure 6.

11. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 2, 4, 6, 8 or 10 according to Figure 6.

12. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 2, 3, 4 or 5 according to Figure 6.

13. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 6, 7, 8, 9 or 10 according to Figure 6.

14. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 5, 6, 7, 8 or 9 according to Figure 6.

15. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 1, 3, 7, 9 or 10 according to Figure 6.

16. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 2, 5, 8, 9 or 10 according to Figure 6.

17. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 3, 4, 5, 6 or 7 according to Figure 6.

18. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 4, 6, 7, 8 or 9 according to Figure 6.

19. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, comprising any one of SEQ ID NOS: 20 to 79 according to Figure 6.

20. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 19 wherein said macromolecule is derived from camel.

21. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 19 wherein said macromolecule is derived from human.

22. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to claims 1 to 19 wherein said macromolecule is derived from mouse.

23. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to claims 1 to 19 wherein said macromolecule is derived from any vertebrate species other than camel, human and mouse.

24. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 23, as an artificial mutant.

25. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 24, as a peptide homologue of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof.

26. A polypeptide comprising a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 25.

27. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 1 to 26, or a peptide according to claim 26 wherein one or more amino acids are derivatized.

28. A method to functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof by replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and optionally replacing the amino acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

29. A method to functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof by replacing the amino acid at position 103 with R, said position determined according to the Kabat numbering.

30. A method to humanize and functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof, said method comprising replacing the amino acid at position 45 with L, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position

44 with G and/or the amino acid at position 47 with W, and replacing of amino acid at position 103 with R, said position determined according to the Kabat numbering

31. A method to humanize and functionalize a heavy chain antibody, a single domain heavy chain antibody, a VH domain, or a fragment thereof, said method comprising replacing the amino acid at position 45 with L, replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position 44 with G and/or the amino acid at position 47 with W, and optionally replacing the amino acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

32. A method according to claim 28 and 29, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from human or mouse.

33. A method according to claim 28 and 29, wherein said heavy chain antibody, single domain heavy chain antibody, VH domain, or fragment thereof is derived from any vertebrate species other than human and mouse.

34. A method to humanize a functional camelid heavy chain antibody, a functional camelid single domain heavy chain, a functional camelid VHH domain or a functional fragment thereof, said method comprising replacing the amino acid at position 45 with L, and optionally replacing the amino acid at position 37 with V and/or the amino acid at position 44 with G and/or the amino acid at position 47 with W, said positions determined by the Kabat numbering.

35. A method to camelize a functional heavy chain antibody, a functional single domain heavy chain, a functional VH domain or a functional fragment thereof, said method comprising replacing the amino acid at position 45 with an amino acid independently chosen from the group consisting of L, V and P, replacing the amino acid at position 103 with an amino acid independently chosen from the group consisting of R, G, K, S and P, and replacing the amino acid at position 37 with F, the amino acid at position 44 with G, the amino acid at position 47 with W, and amino acid at position 103 with R, and optionally replacing the amino acid at position 108 with an amino acid independently chosen from the group consisting of L, Q and R, said positions determined by the Kabat numbering.

36. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtainable by the method of claim 28 to 33 and 35.

37. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtained by the method of claim 28 to 33 and 35.

38. A functional humanized camelid heavy chain antibody, a functional humanized camelid single domain heavy chain, a functional humanized VH domain or a functional fragment thereof, obtainable by the method of claim 34.

39. A functional humanized camelid heavy chain antibody, a functional humanized camelid single domain heavy chain, a functional humanized VH domain or a functional humanized fragment thereof, obtained by the method of claim 34.

40. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 36 to 39, as an artificial mutant.

41. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 36 to 40, as a peptide homologue of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof.

42. A polypeptide comprising a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 36 to 41.

43. A functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, according to any of claims 36 to 41, or a polypeptide according to claim 42 wherein one or more amino acids is derivatized.

44. A library, comprising one or more functional heavy chain antibodies, functional single domain antibodies, functional VH domains, or functional fragments thereof according to any of the claims 36 to 43.

45. A method to make a library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or a functional fragment thereof, comprising the steps:

- introducing a restriction enzyme recognition site in the coding region of the framework 4 region of a VH chain, whereby the cutting site of said restriction enzyme recognition site is situated in the CDR3 region,
- cutting the nucleic acid molecule comprising said coding sequence with said restriction enzyme,

- ligating a double stranded primer to the VH encoding nucleic acid molecule, restoring the CDR3 and so introducing an R amino acid at position 103, said position determined by the Kabat numbering, and
- amplifying the ligated fragments.

46. A method according to claim 45, whereby said restriction enzyme cut is situated within the last two codons of the CDR3 coding region.

47. A method according to claim 45 and 46, whereby said restriction enzyme creates a GA 3' sticky end by cutting before the first nucleotide of the codon coding for amino acid position 101 and after the second nucleotide of codon coding for amino acid position 101 on the complementary strand, said positions determined according to the Kabat numbering.

48. A method according to claims 45 to 47, whereby said restriction enzyme cut is situated within the last codon of the CDR3 coding region.

49. A method according to claims 45 to 48, whereby said restriction enzyme is creating a CA-3' sticky end by cutting before the second nucleotide of codon coding for amino acid position 102 and after the third nucleotide of codon 102 on the complementary strand, said position determined according to the Kabat numbering.

50. The method of claim 45 to 49, whereby said restriction enzyme is chosen from the group consisting of BpmI, Eco57I, BsgI, Smu I, Fau I, Bse RI, and Bfi I.

51. A method to make a library comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, comprising a step of amplification of nucleic acid strands encoding a repertoire of immune or non-immune VHH antibodies, using a

framework 1 specific primer as forward primer, and a back primer which anneals to said nucleic acid strands such that its 3'-terminal three nucleotides are positioned over the codon of the nucleic acid strands which encode amino acid position 103, the reverse-complement of said 3'-terminal three nucleotides encoding R103, K103, Q103, F103, P103, G103 or S103, said position determined according to the Kabat numbering.

52. A method to make a library comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, comprising a step of amplification of nucleic acid encoding a repertoire of immune or non-immune VHH antibodies or fragments thereof, using a framework 1 specific primer, as forward primer, and using one or more of SEQ ID NOs: 80 to 88 according to Figure 6 as back primers.

53. A library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, obtainable by the method according to any of claims 45 to 52.

54. A library, comprising at least one functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, obtained by the method according to any of claims 45 to 52.

55. A library according to claims 53 to 54 wherein the methods use a single domain heavy chain library from human or mouse.

56. A library according to claims 53 to 54 wherein the methods use a single domain heavy chain library from camel.

57. A library according to claims 53 to 54 wherein the methods use a single domain heavy chain library from any vertebrate species other than camel, human or mouse.

58. A heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof, obtained by the method of claim 28 to 35 for use in immunoassays.

59. A recombinant DNA construct useful for the expression of a polypeptide in a cell containing the construct, the construct comprising control sequences which regulate transcription and translation of the said antibody in the cell and a coding sequence regulated by the control sequences, wherein the coding sequence comprises a DNA sequence of at least 21bp in reading frame in that the DNA sequence encodes a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42.

60. A nucleic acid comprising a DNA sequence encoding a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42.

61. A nucleic acid having a nucleotide sequence which is at least 65% identical to the sequence defined in claim 60.

62. A vector comprising a nucleic acid sequence according to any of claims 60 and 61.

63. A host cell comprising an integrated or episomal copy of a nucleic acid molecule according to claims 60 and 61, or a vector according to claim 62.

64. The host cell of claim 63, wherein said host cell is a yeast, bacterial, insect, fungal, plant or mammalian cell.

65. A method for producing a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, comprising:

(a) culturing host cells comprising a nucleic acid according to any of claims 60 and 61, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

66. The use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, or nucleic acid according to any of claims 60 and 61 for the preparation of a medicament.

67. The use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, or nucleic acid according to any of the claims 60 and 61 for the preparation of a medicament for the treatment of a disease related to asthma, rhinoconjunctivitis, allergic disorders, acute allograft rejection, Crohn's disease and ulcerative colitis.

68. A pharmaceutical composition comprising a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, or nucleic acid according to claims 60 and 61, optionally in combination with a suitable excipient.

69. The use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, or nucleic acid according to claims 60 and 61 in the diagnosis of a disease related to asthma, rhinoconjunctivitis, allergic disorders, acute allograft rejection, Crohn's disease and ulcerative colitis.

70. The use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42 for the purification of a protein.

71. The use of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42 for the purification of a protein.

72. A kit for the diagnosis of a pathological condition or a susceptibility to a pathological condition in a subject comprising a nucleic acid according to claims 60 and 61, a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42.

73. A method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of :

(a) determining the presence or absence of a mutation in the nucleic acid according to any of claims 60 and 61, including mutations in the genomic and regulatory sequences of said nucleic acid, in a biological sample, and

(b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or absence of said mutation.

74. A method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of :

(a) determining the presence or amount of the nucleic acid according to claims 60 and 61 or expression of a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42 in a biological sample, and,

(b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of said nucleic acid or expression of said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, functional fragment thereof or polypeptide.

75. A drug screening assay for screening test compounds which interact with a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42, comprising:

(a) combining a functional heavy chain antibody, a functional single domain heavy chain antibody, a functional VH domain, or a functional fragment thereof according to any of claims 1 to 25, 27, 36 to 41, 43 or a polypeptide according to claims 26 and 42 with a test compound, under conditions which allow for interaction of the test compound to said functional heavy chain

antibody, functional single domain heavy chain antibody, functional VH domain, functional fragment thereof or polypeptide, to form a complex, and,

(b) detecting the formation of a complex, in which the ability of the test compound to interact with the said functional heavy chain antibody, functional single domain heavy chain antibody, functional VH domain, or functional fragment thereof, is indicated by the presence of the test compound in the complex.

76. The product or compound identifiable by the assay of any of the claims 75.

77. A nucleic acid comprising the sequence SEQ ID NO: 80 according to Figure 6.
78. A nucleic acid comprising the sequence SEQ ID NO: 81 according to Figure 6.
79. A nucleic acid comprising the sequence SEQ ID NO: 82 according to Figure 6.
80. A nucleic acid comprising the sequence SEQ ID NO: 83 according to Figure 6.
81. A nucleic acid comprising the sequence SEQ ID NO: 84 according to Figure 6.
82. A nucleic acid comprising the sequence SEQ ID NO: 85 according to Figure 6.
83. A nucleic acid comprising the sequence SEQ ID NO: 86 according to Figure 6.
84. A nucleic acid comprising the sequence SEQ ID NO: 87 according to Figure 6.
85. A nucleic acid comprising the sequence SEQ ID NO: 88 according to Figure 6.

86. A nucleic acid having a nucleotide sequence which is at least 65% identical to the sequence defined in claims 77 to 85.

87. The use of a nucleic acid according to claims 77 to 86 in a method to produce one or more functional heavy chain antibodies, functional single domain heavy chain antibodies, functional VH domains, or functional fragments thereof.

Figure 1-1

(A) TEM04

```
  1  caggtgcagc tggtggagtc tgggggaggc ttggtgcagg ctggagggtc
tctgaggctc
      q  v  q   l  v  e   s  g  g  g   l  v  q   a  g  g   s  l  r  l

 61  tcctgtgcag cctctggatt caccttcagt agcgcatgga tgacatgggt
ccgccaggct
      s  c  a   a  s  g   f  t  f  s   s  a  w   m  t  w   v  r  q  a

121  ccagggaagg gactcgagtg ggtcacaagt attgctacgg atgggtccac
ggactatgca
      p  g  k   g  l  e   w  v  t  s   i  a  t   d  g  s   t  d  y  a

181  gactccgtga agggccgatt caccatctcc agagacaatg ccaagaacac
gctgtatctg
      d  s  v   k  g  r   f  t  i  s   r  d  n   a  k  n   t  l  y  l

241  caattaaaca gcctgaacac tgaagacacg gccgtgtatt actgtgcaaa
agatcgttgg
      q  l  n   s  l  n   t  e  d  t   a  v  y   y  c  a   k  d  r  w

301  gggtatgtcg taagaggcca ggggacccag gtcaccgtct cctca
      g  y  v   v  r  g   q  g  t  q   v  t  v   s  s
```

AGA = wild type

TGG = mutant

Figure 1-2

(B) CEA71

```
  1  caggtgcagc tggtggagtc tgggggaggc ttggtgcaac ctgggggggtc
tctgagactc
      q  v  q   l  v  e   s  g  g  g   l  v  q   p  g  g   s  l  r  l

 61  tcctgtgcag cctctggatt caccttcagt agcagctaca tgagctgggt
ccgccaggct
      s  c  a   a  s  g   f  t  f  s   s  s  y   m  s  w   v  r  q  a

121  ccagggaagg ggctggagtg ggtgtccggc attaataccg atggaagttt
cacgcgctat
      p  g  k   g  l  e   w  v  s  g   i  n  t   d  g  s   f  t  r  y

181  gccgactccg tgaagggccg attcaccatc tccagagaca acgccaagaa
cacgctgtat
      a  d  s   v  k  g   r  f  t  i   s  r  d   n  a  k   n  t  l  y

241  ctgcaaatga acagcctgaa atctgaggac acggccctgt attactgtgc
cgtaggcggc
      l  q  m   n  s  l   k  s  e  d   t  a  l   y  y  c   a  v  g  g

301  gggttaggct atggccccag gggccagggg accctggtca ctgtctcctc a
      g  l  g   y  g  p   r  g  q  g   t  l  v   t  v  s   s
```

AGG = wild type

TGG = mutant

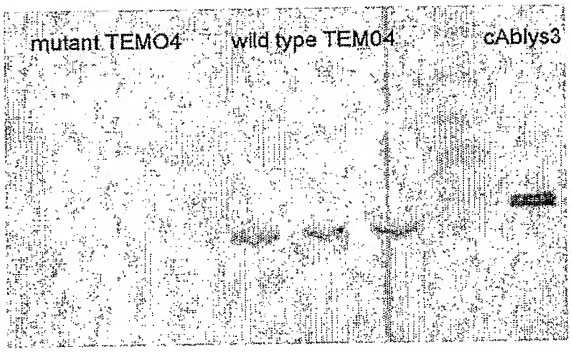

Figure 2A

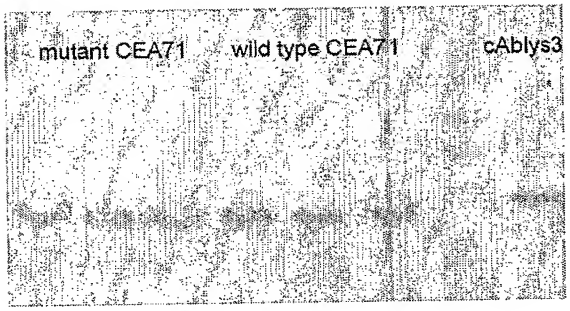

Figure 2B

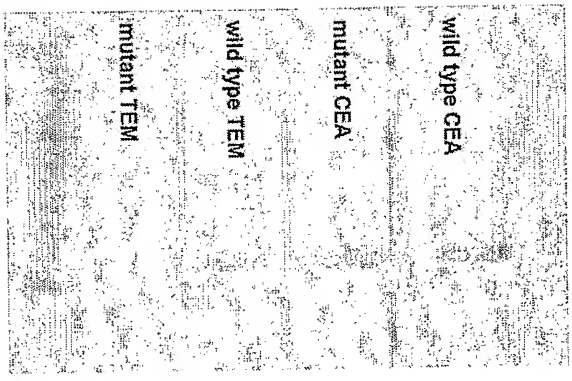

Figure 3

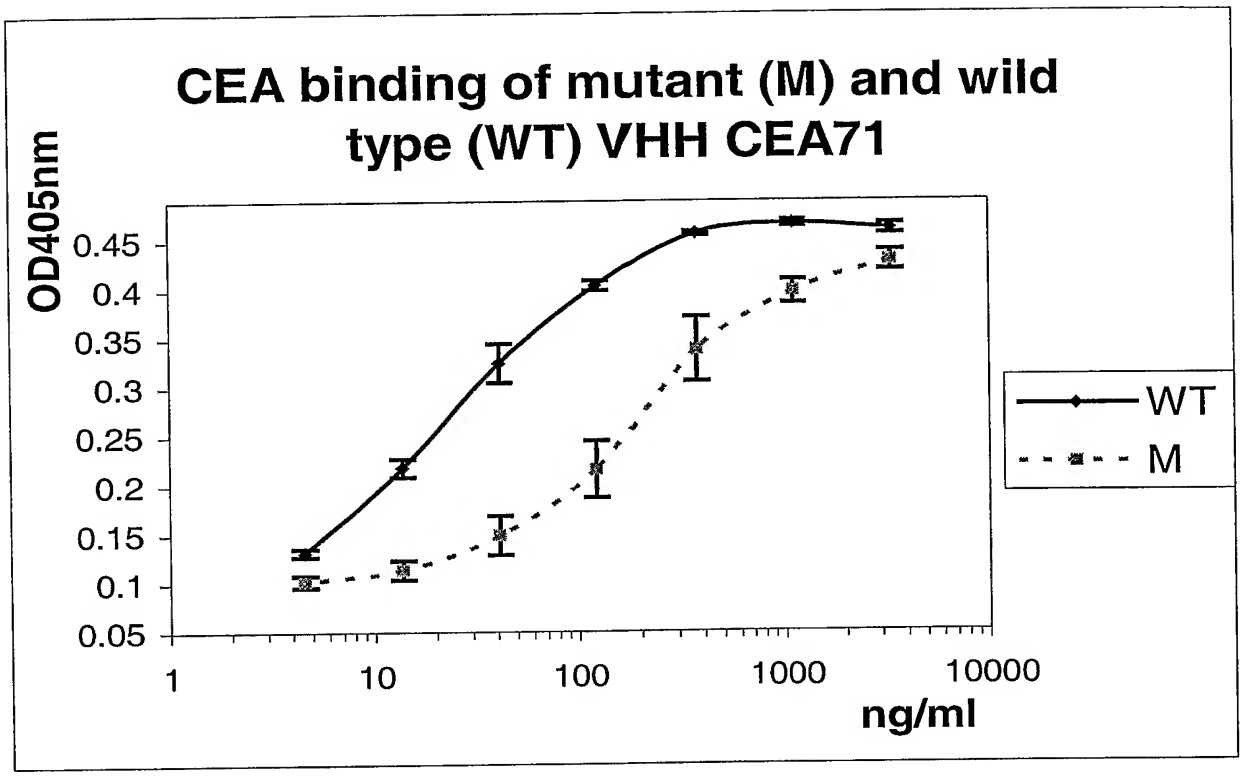
CEA binding of mutant (M) and wild type (WT) VHH CEA71
OD405nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
1
10
100
1000
10000
ng/ml
WT
M

Figure 4

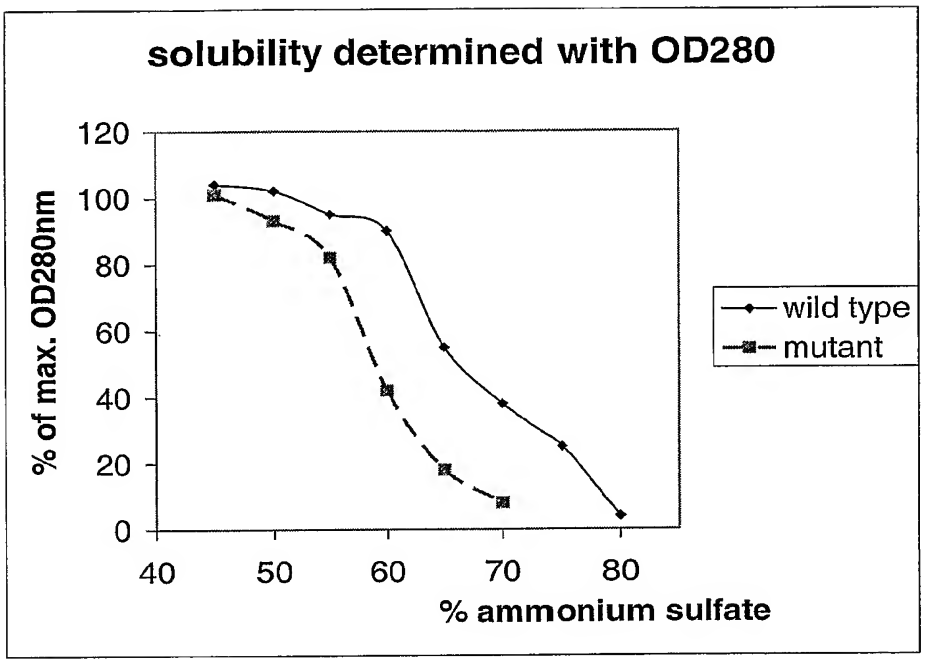

Figure 5A

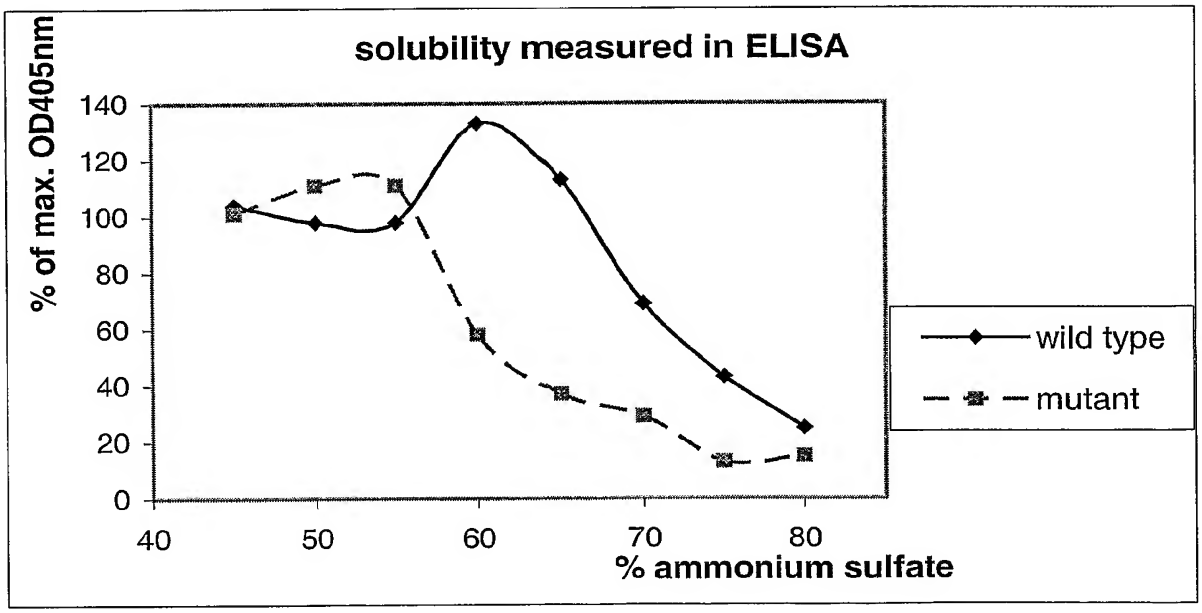

Figure 5B

Figure 6-1

SEQ ID NO:	Position #45	Position #103	Position #108
1	Not charged, and not C	G	Q
2	Not charged, and not C	G	R
3	Not charged, and not C	K	R
4	Not charged, and not C	L	R
5	Not charged, and not C	P	L
6	Not charged, and not C	P	R
7	Not charged, and not C	Q	L
8	Not charged, and not C	Q	R
9	Not charged, and not C	S	L
10	Not charged, and not C	S	R

SEQ ID NO: 13 RGQGTQ
SEQ ID NO: 14 RGKGTQ
SEQ ID NO: 15 VXXXXXXGLXW
SEQ ID NO: 16 LGQGTQVTVSS
SEQ ID NO: 17 QGQGTGVTVSS
SEQ ID NO: 18 PGQGTQVTVSS
SEQ ID NO: 19 SSQGTQVTVSS

SEQ ID NO:	Position #45	Position #103	Position #108
20	P	G	Q
21	V	G	Q
22	S	G	Q
23	L	G	Q
24	M	G	Q
25	W	G	R
26	A	G	R
27	T	G	R
28	G	G	R
29	F	G	R
30	A	K	R
31	F	K	R
32	M	K	R
33	I	K	R
34	P	K	R
35	V	L	R
36	I	L	R
37	F	L	R
38	Y	L	R
39	T	L	R
40	L	P	L

Figure 6-2

41	I	P	L
42	P	P	L
43	V	P	L
44	W	P	L
45	G	P	R
46	L	P	R
47	F	P	R
48	S	P	R
49	M	P	R
50	T	Q	L
51	M	Q	L
52	F	Q	L
53	I	Q	L
54	V	Q	L
55	V	Q	R
56	W	Q	R
57	A	Q	R
58	S	Q	R
59	Y	Q	R
60	I	R	Q
61	G	R	Q
62	W	R	Q
63	P	R	Q
64	A	R	Q
65	P	R	R
66	A	R	R
67	M	R	R
68	V	R	R
69	T	R	R
70	A	S	L
71	I	S	L
72	Y	S	L
73	P	S	L
74	S	S	L
75	T	S	R
76	M	S	R
77	V	S	R
78	A	S	R
79	P	S	R

SEQ ID NO: 80 Primer 1 (R103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/T/C/G)CG -3'

SEQ ID NO: 81 Primer 2 (R103):
5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (C/T)CT-3'

SEQ ID NO: 82 Primer 3 (K103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC GCT GGA GAC GGT GAC CTG GGT CCC CTG GCC (T/C)TT -3'

Figure 6-3

SEQ ID NO: 83 Primer 4 (Q103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GC(C/G) (C/T)TG -3'

SEQ ID NO: 84 Primer 5 (L103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/G/C/T)AG -3'

SEQ ID NO: 85 Primer 6 (F103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC (A/G)AA -3'

SEQ ID NO: 86 Primer 7 (G103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CCC CGG (A/G/C/T)CC -3'

SEQ ID NO: 87 Primer 8 (S103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG (A/G/C/T)GA (A/G/C/T)GA -3'

SEQ ID NO: 88 Primer 9 (P103):
5'- GAG TCA TTC TCG ACT TGC GGC CGC TGA GGA GAC GGT GAC CTG GGT CCC CTG CTG (A/G/C/T)GG -3'